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FILE 'MEDLINE' ENTERED AT 09:22:33 ON 05 MAR 2002

FILE 'AGRICOLA' ENTERED AT 09:22:33 ON 05 MAR 2002

FILE 'CAPLUS' ENTERED AT 09:22:33 ON 05 MAR 2002
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=> s azacytidine
L1 6800 AZACYTIDINE

=> s l1 or (hexamethylene (w) diisocyanate)
L2 16350 L1 OR (HEXAMETHYLENE (W) DIISOCYANATE)

=> l2 and (bacteriophage or phage or phagolysis or lysis)
L2 IS NOT A RECOGNIZED COMMAND
The previous command name entered was not recognized by the system.
For a list of commands available to you in the current file, enter
"HELP COMMANDS" at an arrow prompt (=>).

=> s l2 and (bacteriophage or phage or phagolysis or lysis)
L3 81 L2 AND (BACTERIOPHAGE OR PHAGE OR PHAGOLYSIS OR LYSIS)

=> s l3 and lacto?
L4 0 L3 AND LACTO?

=> dup rem l3
PROCESSING COMPLETED FOR L3
L5 38 DUP REM L3 (43 DUPLICATES REMOVED)

=> d 1- ibib abs
YOU HAVE REQUESTED DATA FROM 38 ANSWERS - CONTINUE? Y/(N):y

L5 ANSWER 1 OF 38 WPIDS COPYRIGHT 2002 DERWENT INFORMATION LTD
ACCESSION NUMBER: 2001-657582 [76] WPIDS
DOC. NO. CPI: C2001-193657
TITLE: Gene complex for reversible cell immortalization, useful for expanding cells for cell replacement therapy of e.g. neurodegenerative disease, contains removable immortalizing gene.
DERWENT CLASS: B04 D16
INVENTOR(S): KANDOLF, R; KUEPPER, J; KUHN, A
PATENT ASSIGNEE(S): (UYTU-N) UNIV TUEBINGEN EBERHARD-KARLS
COUNTRY COUNT: 23
PATENT INFORMATION:

PATENT NO	KIND	DATE	WEEK	LA	PG
DE 10019195	A1	20011025	(200176)*		10
WO 2001078757	A2	20011025	(200176)	GE	

RW: AT BE CH CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE TR
W: AU CA JP US

APPLICATION DETAILS:

PATENT NO	KIND	APPLICATION	DATE
DE 10019195	A1	DE 2000-10019195	20000417
WO 2001078757	A2	WO 2001-EP2967	20010315

PRIORITY APPLN. INFO: DE 2000-10019195 20000417
AN 2001-657582 [76] WPIDS
AB DE 10019195 A UPAB: 20011227
NOVELTY - Gene complex (A) for reversible immortalization of cells, comprising an immortalizing gene region (B), two flanking sequences (FS)

around (B) that function as sites for homologous recombination, and at least one promoter upstream of (B), is new. (B) contains at least one resistance gene (RG), an immortalizing gene (IG) and preferably a suicide gene (SG).

DETAILED DESCRIPTION - INDEPENDENT CLAIMS are also included for the following:

(1) gene complex (A1) for immunomodulation of cells comprising first immunomodulatory gene region (C1), expression of which inhibits the function of MHC (major histocompatibility complex) Class I on cells, a second immunomodulatory gene region (C2) expression of which inactivates NK (natural killer) cells, and RG;

(2) producing cells (D) by preparing organ-related cells, immortalizing them, expanding and then reversing the immortalization;

(3) cells produced by the method of (2);

(4) pharmaceutical composition containing cells of (3);

(5) plasmid or viral vector that contains (A) or (A1);

(6) transplant material that contains cells of (3); and

(7) kit containing (A) or (A1).

ACTIVITY - Cardiant; antiParkinsonian; osteopathic; hepatotropic; antiinflammatory.

No biological data is given.

MECHANISM OF ACTION - Cell and protein replacement.

USE - (A) is used to immortalize cells so that these can be expanded in culture and, after reversal of immortalization, used to produce transplants for organ regeneration (for treating myocardial, neurodegenerative, bone and liver diseases, e.g. infarction, Parkinson's disease, osteoporosis or chronic liver inflammation), also for treatment of chronic diseases. The cells may also be used for extracellular preparation of tissues, e.g. seeded into collagen/fibronectin biomaterials to produce e.g. cardiac or venous valves.

ADVANTAGE - The construct provides immunologically and clinically tolerable cells inexpensively and in practically unlimited quantities. Allogenic cells can be rendered immunotolerant by transforming with a modulatory gene construct.

Dwg.0/2

L5 ANSWER 2 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1999:34409 CAPLUS
DOCUMENT NUMBER: 130:91255
TITLE: Covalent labeling of nucleic acids with proteins using fusion proteins with DNA methylases
INVENTOR(S): Epstein, David M.
PATENT ASSIGNEE(S): The Scripps Research Institute, USA
SOURCE: U.S., 90 pp.
CODEN: USXXAM
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 5856090	A	19990105	US 1994-305764	19940909

AB A method of using sequence-specific DNA binding proteins, such as DNA methylases, as intermediates in the conjugation of nucleic acids with protein labels such as immunol. determinants is described. The polypeptide label is manufd. as a fusion protein with a DNA methylase that binds in vivo to a cytidine suicide analog when present in a nucleotide sequence. Expression vectors for the manuf. of these fusion proteins are described. If the plasmids contains a methylase recognition site contg. the cytidine suicide analog, then the peptide label can be attached to the plasmid. The method is intended for use in screening display libraries because conjugation of the protein to the plasmid encoding it allows immediate recovery of DNA encoding a determinant. Use of an MspI methylase fusion product with glutathione-S-transferase to label **azacytidine** or fluorocytidine-labeled plasmid is demonstrated.

REFERENCE COUNT: 29 THERE ARE 29 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L5 ANSWER 3 OF 38 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 95181817 MEDLINE
DOCUMENT NUMBER: 95181817 PubMed ID: 7533191
TITLE: Mechanism of drug-induced lupus. I. Cloned Th2 cells modified with DNA methylation inhibitors in vitro cause autoimmunity in vivo.
AUTHOR: Yung R L; Quddus J; Chrisp C E; Johnson K J; Richardson B C
CORPORATE SOURCE: Department of Internal Medicine, University of Michigan, Ann Arbor.

CONTRACT NUMBER: AR07080 (NIAMS)
AR20557 (NIAMS)
AR42525 (NIAMS)
SOURCE: JOURNAL OF IMMUNOLOGY, (1995 Mar 15) 154 (6) 3025-35.
Journal code: IFB; 2985117R. ISSN: 0022-1767.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199504
ENTRY DATE: Entered STN: 19950419
Last Updated on STN: 19970203
Entered Medline: 19950406

AB Treating activated CD4+ T cells with DNA methyltransferase inhibitors modifies gene expression and induces autoreactivity. Adoptive transfer of viable polyclonal autoreactive cells causes a lupus-like disease, most likely because of one or more effector functions expressed by the autoreactive cells. However, the number of potential effector mechanisms expressed by polyclonal cells is large. To more readily identify responsible mechanisms, we asked if autoimmunity can be induced by using the conalbumin-reactive, cloned Th2 cell line D10.G4.1, treated with 5-**azacytidine** (5-azaC) or procainamide (Pca). Treated, but not untreated, cells responded to syngeneic APCs without Ag, overexpressed LFA-1, spontaneously lysed syngeneic macrophages, and secreted relatively large amounts of IL-6, small amounts of IL-4, and no detectable IL-2 nor IFN-gamma. Adoptive transfer of treated, but not untreated, cells induced a severe immune complex glomerulonephritis, pulmonary alveolitis, central nervous system abnormalities including fibrinoid necrosis, karyorrhexis, and meningitis, and bile duct proliferation with periportal inflammatory cell infiltration resembling primary biliary cirrhosis. Anti-ssDNA, anti-dsDNA, and anti-histone Abs were also found. These experiments demonstrate that modification of this cloned T cell line with DNA methyltransferase inhibitors is sufficient to cause an autoimmune disease, with features of lupus as well as autoimmune liver disease. The results also raise the possibility that macrophage **lysis**, IL-6 secretion, and LFA-1 overexpression could contribute to the disease process. This system may be useful in testing the role of these and other pathologic mechanisms in the development of specific autoimmune lesions.

L5 ANSWER 4 OF 38 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

ACCESSION NUMBER: 1993:370863 BIOSIS
DOCUMENT NUMBER: PREV199396056538 ,
TITLE: Modelling the growth kinetics of Phanerochaete chrysosporium in submerged static culture.
AUTHOR(S): Barclay, Clayton D.; Legge, Raymond L. (1); Farquhar, Grahame F.
CORPORATE SOURCE: (1) Biochemical Eng. Group, Dep. Chemical Engineering, Univ. Waterloo, 200 Univ. Avenue West, Waterloo, Ontario, Canada N2L 3G1
SOURCE: Applied and Environmental Microbiology, (1993) Vol. 59, No. 6, pp. 1887-1892.
ISSN: 0099-2240.
DOCUMENT TYPE: Article
LANGUAGE: English

AB The potential commercial application of Phanerochaete chrysosporium requires methods for quantitatively predicting growth and substrate utilization. The growth kinetics of P. chyrsosporium INA-12 (CNCM I-398) were investigated and modelled under nonlimiting nitrogen and carbon conditions in submerged static culture. This strain, unlike other strains, does not require nutrient limitation for induction of lignin peroxidase. Maximum levels of lignin peroxidase activity were reached 7 days after culture initiation, when almost 80% of the initial glycerol and 70% of the initial nitrogen were still present. Lignin peroxidase levels then decreased while biomass levels increased until about day 14. The ratio of cell dry weight to wet weight was constant until the maximum biomass concentration was achieved, after which there was a decrease in the water content. The change in this ratio reflects cell **lysis** as it correlated with increased concentrations of nitrogen in the media, arising from cell leakage. The suitability of four growth models to predict growth, and in some cases glycerol consumption, was evaluated. A simple linear model and the Emerson model performed poorly for the early stages of growth, while a modified Williams model and that Monod model predicted substrate and biomass concentrations equally well. All models will predict biomass concentrations during the active growth phase, but they should not be used to predict biomass concentrations after the stationary growth phase, when cell **lysis** becomes significant.

L5 ANSWER 5 OF 38 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

ACCESSION NUMBER: 1993:388736 BIOSIS
DOCUMENT NUMBER: PREV199396064036
TITLE: HLA-E is the only class I gene that escapes CpG methylation and is transcriptionally active in trophoblast-derived human cell line JAR.
AUTHOR(S): Boucraut, Jose; Guillaudeux, Thierry; Alizadeh, Mehdi; Boretto, Joelle; Chimini, Giovanna; Malecaze, Francois; Semana, Gilbert; Fauchet, Renee; Pontarotti, Pierre; Le Bouteiller, Philippe (1)
CORPORATE SOURCE: (1) INSERM U100, CHU Purpan, F-31052 Toulouse Cedex France
SOURCE: Immunogenetics, (1993) Vol. 38, No. 2, pp. 117-130. ISSN: 0093-7711.
DOCUMENT TYPE: Article
LANGUAGE: English
AB Polymorphic as well as HLA-F and -G genes are repressed in the human cell line JAR, derived from a tumor of trophoblast origin. By contrast, the HLA-E gene as well as the non-HLA novel coding-sequence, R1, located 5' to HLA-E, both remain transcriptionally active. We first demonstrated the role of DNA methylation in the repression of class I genes (except HLA-E) in JAR by the use of the 5-**Azacytidine** demethylating agent. Following treatment, JAR clones reexpressed polymorphic class I transcripts and cell surface alpha chains. Using methylation-sensitive rare cutter enzymes on JAR genomic DNA, followed by classical or pulse field gel electrophesis and hybridization with HLA locus-specific probes, we found methylated CpG islands in the 5' region of all class I genes, except for HLA-E. These results, establishing an inverse relationship between stats of methylation and transcriptional activity within the MHC class I chromosomal region in JAR, and the observations that the HLA-E and R1 genes were ubiquitously expressed, suggest that the HLA-E chromosomal domain might have functional importance including the presence of housekeeping genes.

L5 ANSWER 6 OF 38 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 93315670 MEDLINE
DOCUMENT NUMBER: 93315670 PubMed ID: 7686923
TITLE: Treating activated CD4+ T cells with either of two distinct DNA methyltransferase inhibitors, 5-**azacytidine** or procainamide, is sufficient to cause a lupus-like disease in syngeneic mice.
AUTHOR: Quddus J; Johnson K J; Gavalchin J; Amento E P; Chrisp C E; Yung R L; Richardson B C
CORPORATE SOURCE: Department of Internal Medicine, University of Michigan, Ann Arbor 48109.
CONTRACT NUMBER: AI-25526 (NIAID)
AR-20557 (NIAMS)
P40 RR-00200 (NCRR)
+
SOURCE: JOURNAL OF CLINICAL INVESTIGATION, (1993 Jul) 92 (1) 38-53. Journal code: HS7; 7802877. ISSN: 0021-9738.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
ENTRY MONTH: 199308
ENTRY DATE: Entered STN: 19930820
Last Updated on STN: 19960129
Entered Medline: 19930812
AB Human antigen-specific CD4+ T cells become autoreactive after treatment with various DNA methylation inhibitors, including 5-**azacytidine**, procainamide, and hydralazine. This suggests a mechanism that could contribute to the development of some forms of autoimmunity. In this report we have asked whether T cells treated with DNA methylation inhibitors can induce autoimmunity. Murine CD4+ T cells were treated with 5-**azacytidine** or procainamide and were shown to respond to syngeneic antigen-presenting cells, similar to CD4+ human T cell clones treated with these drugs. Functional characterization demonstrated that cells treated with either drug spontaneously lysed syngeneic macrophages and secreted IL-4, IL-6, and IFN-gamma. Adoptive transfer of 5-**azacytidine**- or procainamide-treated cells into unirradiated syngeneic recipients induced an immune complex glomerulonephritis and IgG anti-DNA and antihistone antibodies. These experiments demonstrate that T cells treated with either of two distinct DNA methyltransferase inhibitors are sufficient to induce a lupus-like disease. It is possible that the **lysis** of macrophages, together with the release of cytokines promoting B cell differentiation, contributes to the autoantibody production and immune complex deposition. These results suggest that environmental agents that inhibit DNA methylation could interact with T cells in vivo to produce a lupus-like illness, a mechanism that could have

relevance to drug-induced and idiopathic lupus.

L5 ANSWER 7 OF 38 MEDLINE DUPLICATE 3
ACCESSION NUMBER: 92324302 MEDLINE
DOCUMENT NUMBER: 92324302 PubMed ID: 1378022
TITLE: Expression of the complement regulatory proteins CD21, CD55 and CD59 on Burkitt lymphoma lines: their role in sensitivity to human serum-mediated **lysis**.
AUTHOR: Kuraya M; Yefenof E; Klein G; Klein E
CORPORATE SOURCE: Department of Tumor Biology, Karolinska Institute, Stockholm, Sweden.
CONTRACT NUMBER: 2RO1 CA 25250-10 (NCI)
SOURCE: EUROPEAN JOURNAL OF IMMUNOLOGY, (1992 Jul) 22 (7) 1871-6.
Journal code: EN5; 1273201. ISSN: 0014-2980.
PUB. COUNTRY: GERMANY: Germany, Federal Republic of
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199208
ENTRY DATE: Entered STN: 19920821
Last Updated on STN: 19980206
Entered Medline: 19920811

AB On a panel of nine human B cell lines we showed that the expression of the complement regulatory factors complement receptor type 2 (CR2; CD21), decay-accelerating factor, (DAF; CD55) and homologous restriction factor (HRF20, CD59) is not correlated. All lines expressed DAF, six lines carried detectable amounts of CR2 and three carried HRF20. Upon incubation in human serum, under conditions which allowed the activation of complement through the alternative pathway, the CR2-carrying lines bound C3 fragments and two of them (Ramos and one of its two sublines) were damaged. These two lines had the lowest DAF expression, less than 50% of the cells reacted with the IA10 monoclonal antibody. By modulating the expression of the complement regulatory molecules, the lytic sensitivity of the B cell lines could be altered. Blockade of DAF on the HRF20-, CR2+ lines with the specific monoclonal antibodies increased their sensitivity to **lysis** by human serum. With the DAF- and HRF20+ cells significant lytic effect was obtained only when they were pretreated with both of the specific antibodies. Interferon-gamma or tumor necrosis factor-alpha treatment elevated the amount of CR2 on the low-CR2 expressor line (Ramos/HR1K) which thereafter bound higher amounts of C3 fragments and was lysed when incubated in human serum. This line had relatively low DAF level and lacked HRF20. The cytokine treatment did not alter the expression of these molecules. The CR2+ Ramos and the CR2- Rael cells were treated with 5-**azacytidine** which induced HRF20 and increased DAF expression. In parallel with this change Ramos cells became resistant to C-mediated **lysis**. The experiments with the panel of human B cell lines showed thus that cytolysis through activation of complement in homologous serum can be regulated at several steps by cell surface molecules. While expression of CR2 was required for C3 fixation, DAF and HRF20 inhibited **lysis**. By independent modulation of the quantities of these molecules, cells acquired or lost their sensitivity.

L5 ANSWER 8 OF 38 MEDLINE DUPLICATE 4
ACCESSION NUMBER: 92373513 MEDLINE
DOCUMENT NUMBER: 92373513 PubMed ID: 1380552
TITLE: Increase in susceptibility to EcoRII restriction of **bacteriophage** lambda produced by propagation on host cells growing in 5-**azacytidine**: a new in-vivo method for demonstration of DNA-methylation inhibition.
AUTHOR: Radnedge L; Pinney R J
CORPORATE SOURCE: Department of Pharmaceutics, School of Pharmacy, University of London, UK.
SOURCE: JOURNAL OF PHARMACY AND PHARMACOLOGY, (1992 Mar) 44 (3) 266-8.
Journal code: JNR; 0376363. ISSN: 0022-3573.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199209
ENTRY DATE: Entered STN: 19921009
Last Updated on STN: 19960129
Entered Medline: 19920918

AB The efficiency of plating on EcoRII-restricting cells of **bacteriophage** lambda vir propagated on an Escherichia coli K-12 dcm+ host decreased with increase in concentration of 5-**azacytidine** (5-azaC) in the propagating medium. This illustrates,

in-vivo, the inhibition of DNA-cytosine methylation induced by 5-azaC and provides a simple system for the detection of DNA-methylation inhibitors.

L5 ANSWER 9 OF 38 MEDLINE DUPLICATE 5
ACCESSION NUMBER: 91199100 MEDLINE
DOCUMENT NUMBER: 91199100 PubMed ID: 1849793
TITLE: Two discrete types of tumor necrosis factor-resistant cells derived from the same cell line.
AUTHOR: Vanhaesebroeck B; Van Bladel S; Lenaerts A; Suffys P; Beyaert R; Lucas R; Van Roy F; Fiers W
CORPORATE SOURCE: Laboratory of Molecular Biology, State University, Gent, Belgium.
SOURCE: CANCER RESEARCH, (1991 May 1) 51 (9) 2469-77.
Journal code: CNF; 2984705R. ISSN: 0008-5472.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199105
ENTRY DATE: Entered STN: 19910607
Last Updated on STN: 19970203
Entered Medline: 19910517

AB From the murine fibrosarcoma cell line L929s, which is sensitive to tumor necrosis factor (TNF)-mediated cell **lysis**, two discrete types of TNF-resistant variants were derived by TNF selection. Cells of the first type (named L929r1) were not sensitized to TNF cytotoxicity by cotreatment with either inhibitors of protein or RNA synthesis, or gamma-interferon, despite the presence of a functional gamma-interferon response. L929r1 constitutively produced TNF in the supernatant and expressed membrane-bound TNF, which was not bound to the TNF receptor. In fact, TNF receptors could not be demonstrated on L929r1 cells, not even after low pH treatment and/or incubation with antiserum to TNF. L929r1 exhibited a stable TNF-resistant phenotype in the absence of further TNF selection. No evidence could be obtained that TNF acted as an autocrine growth factor for these cells. L929r2, the second type of TNF-resistant L929 cells, became sensitive to TNF **lysis** in the presence of RNA or protein synthesis inhibitors, or in the presence of gamma-interferon. TNF induced the secretion of interleukin 6 in these cells, additionally showing that functional TNF signaling in these cells indeed takes place, but does not lead to cell **lysis** under normal conditions. L929r2 did not produce TNF, also not upon stimulation with exogenous TNF. The number and binding affinity of TNF receptors were not consistently different between L929s and L929r2 cells. In the absence of further TNF selection, L929r2 gradually reverted to TNF sensitivity. This sensitivity was not reversible to TNF resistance by the gene-regulatory agents 5-**azacytidine** or sodium butyrate. Treatment with these agents also did not affect the TNF sensitivity of L929s cells nor the TNF resistance of L929r1 and L929r2 cells. In summary, our results suggest the existence among cells of the same cell line of discrete mechanisms for acquisition of resistance to TNF-mediated cell **lysis**.

L5 ANSWER 10 OF 38 MEDLINE DUPLICATE 6
ACCESSION NUMBER: 90272407 MEDLINE
DOCUMENT NUMBER: 90272407 PubMed ID: 1693420
TITLE: Development of a short-term, in vivo mutagenesis assay: the effects of methylation on the recovery of a lambda **phage** shuttle vector from transgenic mice.
AUTHOR: Kohler S W; Provost G S; Kretz P L; Dycaico M J; Sorge J A; Short J M
CORPORATE SOURCE: Stratagene, La Jolla, CA 92037.
CONTRACT NUMBER: 1R01 ES04728-01A1 (NIEHS)
2R44 ES04484-02 (NIEHS)
SOURCE: NUCLEIC ACIDS RESEARCH, (1990 May 25) 18 (10) 3007-13.
Journal code: O8L; 0411011. ISSN: 0305-1048.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199007
ENTRY DATE: Entered STN: 19900810
Last Updated on STN: 19960129
Entered Medline: 19900711

AB Transgenic mice suitable for the in vivo assay of suspected mutagens at the chromosome level have been constructed by stable integration of a lambda **phage** shuttle vector. The shuttle vector, which contains a beta-galactosidase (beta-gal) target gene, can be rescued from genomic DNA with in vitro packaging extracts. Mutations in the target gene are detected by a change in lambda **phage** plaque color on indicator

agar plates. Initial rescue efficiencies of less than 1 plaque forming unit (pfu)/100 micrograms of genomic DNA were too low for mutation analysis. We determined the cause of the low rescue efficiencies by examining primary fibroblast cultures prepared from fetuses of lambda transgenic animals. The rescue efficiency of 5-**azacytidine** -treated cells increased 50-fold over non-treated controls indicating that methylation was inhibiting rescue. The inhibitory role of methylation was supported by the observation that mcr deficient E. coli plating strains and mcr deficient lambda packaging extracts further improved lambda rescue efficiency. Present rescue efficiencies of greater than 2000 pfu/copy/micrograms of genomic DNA represent a 100,000-fold improvement over initial rescue efficiencies, permitting quantitative mutational analysis. The background mutagenesis rate was estimated at 1 x 10(-5) in two separate lineages. Following treatment with the mutagen N-ethyl-N-nitrosourea (EtNU), a dose dependent increase in the mutation rate was observed in DNA isolated from mouse spleen, with significant induction also observed in mouse testes DNA.

L5 ANSWER 11 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1991:201194 CAPLUS
DOCUMENT NUMBER: 114:201194
TITLE: The use of lambda **phage** shuttle vectors in transgenic mice for development of a short term mutagenicity assay
AUTHOR(S): Short, Jay M.; Kohler, Steven W.; Provost, G. Scott; Feick, Annabeth; Kretz, Patricia L.
CORPORATE SOURCE: Stratagene Cloning Syst., La Jolla, CA, 92037, USA
SOURCE: Prog. Clin. Biol. Res. (1990), 340A(Mutat. Environ., Pt. A), 355-67
CODEN: PCBRD2; ISSN: 0361-7742
DOCUMENT TYPE: Journal
LANGUAGE: English
AB This article describes the development of transgenic mouse lines that contain an integrated lambda **phage** shuttle vector which can be rapidly and efficiently recovered from genomic DNA by in vitro packaging. The effects of eukaryotic methylation on lambda rescue efficiency are discussed as well as the approaches taken to circumvent this problem. The transgenic mice, lambda packaging exts., and Escherichia coli strains generated in this study should lead to the development of a sensitive short-term in vivo assay for assessing the mutagenicity of suspected genotoxic substances.

L5 ANSWER 12 OF 38 MEDLINE DUPLICATE 7
ACCESSION NUMBER: 91208004 MEDLINE
DOCUMENT NUMBER: 91208004 PubMed ID: 2128468
TITLE: Transfection of the CD8 alpha gene restores specific target cell **lysis**: factors that determine the function and the expression of CD8 in a cytotoxic T cell clone.
AUTHOR: Schmidt-Ullrich R; Eichmann K
CORPORATE SOURCE: Max-Planck-Institut fur Immunobiologie, Freiburg, FRG.
SOURCE: INTERNATIONAL IMMUNOLOGY, (1990) 2 (3) 247-56.
Journal code: AY5; 8916182. ISSN: 0953-8178.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199105
ENTRY DATE: Entered STN: 19910616
Last Updated on STN: 19910616
Entered Medline: 19910529
AB We investigated the functional role of CD8 and the control of CD8 expression in a constitutively activated murine cytotoxic T cell clone (C196), in CD8 deficient variants of this clone, and in cell lines derived by transfecting such variants with the CD8 alpha (Lyt-2) gene. CD8 deficient variants of C196 are deficient in specific target cell **lysis** but retain the ability to perform anti-CD3 induced cytolytic function. Following transfection with the Lyt-2 gene, specific target cell **lysis** was restored in some but not all CD8 positive transfectants whereas no alteration of anti-CD3 induced **lysis** was observed. All CD8 deficient variants studied lost the surface expression of both Lyt-2 and Lyt-3 polypeptide chains. However, while Lyt-2 mRNA was abolished as well, typical CD8 deficient variants retained wild type levels at Lyt-3 mRNA. Moreover, a low level of cytoplasmic Lyt-3 protein was demonstrable. Following transfection with the Lyt-2 gene, both Lyt-2 and Lyt-3 polypeptide chains reappeared on the membrane. In one atypical CD8 deficient variant that carries a mutated Lyt-3 gene and fails to express Lyt-3 mRNA, Lyt-2 transfection causes membrane-reappearance of Lyt-2 only. These results may reflect the occurrence on normal T cells of

Lyt-2/Lyt-3 heterodimers and of Lyt-2/Lyt-2 homodimers, whereas surface expression of Lyt-3 alone has not been observed. In CD8 deficient variant, a particular restriction site 5' at the Lyt-2 gene is methylated which is undermethylated in the wild type C196. Culture of such variants in 5-**azacytidine** partially restored CD8 expression. This suggests a negative correlation of Lyt-2 transcription with site-specific DNA methylation in the C196 system. However, results on T cells unrelated to C196 suggests that the site whose methylation appears to be critical in C196 is not responsible for Lyt-2 transcription in all T cells.

L5 ANSWER 13 OF 38 MEDLINE DUPLICATE 8
ACCESSION NUMBER: 90261332 MEDLINE
DOCUMENT NUMBER: 90261332 PubMed ID: 2140484
TITLE: Tumor necrosis factor mediated cytolysis requires the adenovirus E1a protein but not the transformed phenotype.
AUTHOR: Rodrigues M; Dion P; Sircar S; Weber J M
CORPORATE SOURCE: Department of Microbiology, Faculty of Medicine Centre Hospitalier, Universitaire, Sherbrooke, Quebec, Canada.
SOURCE: VIRUS RESEARCH, (1990 Mar) 15 (3) 231-6.
Journal code: X98; 8410979. ISSN: 0168-1702.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199006
ENTRY DATE: Entered STN: 19900720
Last Updated on STN: 19900720
Entered Medline: 19900626
AB Adenovirus transformed cells are susceptible to **lysis** by human recombinant tumor necrosis factor (TNF). This susceptibility correlates with the presence of E1a in these cells. A flat revertant cell line which expresses a biologically functional E1a but not the transformed phenotype was nevertheless susceptible to TNF. However, flat revertants retransformed by 5-**azacytidine**, without concomitant reactivation of E1a, were resistant to TNF-alpha. This result suggests TNF susceptibility is not transformation but E1a dependent. To study the mechanism of cytolysis in these cell lines, we examined the possibility that changes in the transcription of E1a were brought about by TNF, as it was reported in the case of a c-myc transformed cell line. The results showed that TNF did not affect either E1a or c-myc transcription in our cells during the development of the cytotoxic response.

L5 ANSWER 14 OF 38 WPIDS COPYRIGHT 2002 DERWENT INFORMATION LTD
ACCESSION NUMBER: 1989-246864 [34] WPIDS
DOC. NO. CPI: C1989-110410
TITLE: Erwina carotovora bacterial culture **phage lysis** prevention - involves introduction of 6-aza-cytidine into culture fluid in specified concn..
DERWENT CLASS: B04 D16
INVENTOR(S): ALEKSEEVA, I V; CHERNETSKI, V P; KISHKO, Y A G
PATENT ASSIGNEE(S): (AUMO-R) AS UKR MOLECULAR BIOL; (MICR-R) MICROBIOL VIRUS
COUNTRY COUNT: 1
PATENT INFORMATION:

PATENT NO	KIND	DATE	WEEK	LA	PG
SU 1439121	A	19881123	(198934)*		2

APPLICATION DETAILS:

PATENT NO	KIND	APPLICATION	DATE
SU 1439121	A	SU 1985-3880275	19850109

PRIORITY APPLN. INFO: SU 1985-3880275 19850109
AN 1989-246864 [34] WPIDS
AB SU 1439121 A UPAB: 19930923
Phagolysis of Erwinia carotovora bacterial cultures can be prevented by introducing a protective substance comprising 6-**azacytidine** into the culture fluid. The concn. of 6-**azacytidine** in the culture fluid is typically 1-50 micro.g/ml.
Tests show that the spontaneous prodn. of **phage** particles in samples of the treated cultures is almost completely suppressed in the presence of 6-**azacytidine**, compared with a control, whereas the density of the cell population is not reduced significantly. When **hexamethylene diisocyanate** (used in amt. 0.1 vol.%) is used to prevent bacterial **lysis** (known method), almost complete

suppression of the growth of bacteria (L-asparaginase producers) is observed.
USE/ADVANTAGE - In the microbiological industry for prevention of **phagolysis** of bacterial cultures producing biologically active cpds. In Erwinia carotovora culturing, toxicity is reduced.
Bul.43/23.11.88
0/0

L5 ANSWER 15 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1986:221671 CAPLUS
DOCUMENT NUMBER: 104:221671
TITLE: Procedure for the fixation of biological molecules on a support
INVENTOR(S): Tchen, Paul
PATENT ASSIGNEE(S): Institut National de la Sante et de la Recherche Medicale (INSERM), Fr.
SOURCE: Fr. Demande, 11 pp.
CODEN: FRXXBL
DOCUMENT TYPE: Patent
LANGUAGE: French
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
FR 2567133	A1	19860110	FR 1984-10831	19840706

AB A procedure is described for the irreversible fixation of biol. mols. (e.g., nucleic acid, protein) on a support (e.g., nylon) for biol. anal. and purifn. The procedure uses a compd. with a C4-12 alkyl chain having 2 identical or different functional groups (e.g., carbodiimide, halo, isocyanate) as a bridging agent capable of forming phys. or chem. bonds with free functional groups (e.g., OH, NH2, CO2H) present on the biol. mol. and support. For example, the DNA of .lambda. **phage** was adsorbed on a nylon support filter and rinsed with citrate buffer (10 mM, pH 7.0). The filter was incubated with a soln. of 1,6-diisocyanatohexane (0.07%) contg. 0.3% DMF in citrate buffer (10 mM, pH 7.0) for 2 h. The efficiency of fixation was evaluated using radioactive .lambda. **phage** DNA. The filter obtained was incubated with a dehybridization soln. (10 mM Tris buffer, pH 8.0; 2 mM EDTA) for 5 h at 65.degree.. The remaining DNA on the filter was detd. by autoradiog. The filter prepd. by this fixation method gave intense signals compared to one that was not treated with 1,6-diisocyanatohexane.

L5 ANSWER 16 OF 38 MEDLINE DUPLICATE 9
ACCESSION NUMBER: 87032514 MEDLINE
DOCUMENT NUMBER: 87032514 PubMed ID: 3490445
TITLE: Immunoresistant metastatic tumor variants can re-express their tumor antigen after treatment with DNA methylation-inhibiting agents.
AUTHOR: Altevogt P; von Hoegen P; Schirrmacher V
SOURCE: INTERNATIONAL JOURNAL OF CANCER, (1986 Nov 15) 38 (5) 707-11.
Journal code: GQU; 0042124. ISSN: 0020-7136.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198612
ENTRY DATE: Entered STN: 19900302
Last Updated on STN: 19900302
Entered Medline: 19861211

AB Immuno-escaping variants which arise during metastasis of ESb lymphoma cells in syngeneic DBA/2 mice have been shown to exhibit selective resistance to **lysis** by ESb-specific cytotoxic T-lymphocytes (CTL). The immuno-resistant variants present no changes in the expression of H-2Kd molecules which appear to be the restricting elements for ESb-specific CTL. We now show that treatment of clonal immuno-resistant ESb variant cells with MNNG or 5'**azacytidine** can restore the sensitivity to tumor-specific CTL **lysis** in a high percentage of cloned progenitor cells. The acquisition of susceptibility to **lysis** by these clones is most likely due to re-expression of ESb-type tumor antigens because such cells regain the capacity to compete with original 51Cr-labelled ESb cells for **lysis** by ESb-specific CTL, and regain the capacity to induce ESb-specific CTL in vivo. Our data suggest that the immuno-resistant variants are not cellular mutants but rather gene regulatory variants. This could explain: their high frequency of occurrence during metastasis; the relative stability of the variant phenotype; and the reversibility observed after the use of

DNA-demethylating and gene-activating drugs like 5'-**azacytidine** or MNNG.

L5 ANSWER 17 OF 38 MEDLINE
ACCESSION NUMBER: 86257242 MEDLINE
DOCUMENT NUMBER: 86257242 PubMed ID: 2425255
TITLE: 5-**Azacytidine**: survival and induction of the SOS response in Escherichia coli K-12.
AUTHOR: Barbe J; Gibert I; Guerrero R
SOURCE: MUTATION RESEARCH, (1986 Jul) 166 (1) 9-16.
Journal code: NNA; 0400763. ISSN: 0027-5107.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198608
ENTRY DATE: Entered STN: 19900321
Last Updated on STN: 20000303
Entered Medline: 19860806

AB Survival and induction of the SOS system by 5-**azacytidine**, an analog of cytidine, were studied in Escherichia coli K-12. This compound did not produce any effect on the viability of dcm and dam dcm mutants. Furthermore, recA430 and lexA1 strains (both mutations interfere with LexA repressor cleavage but not recombination proficiency) were more resistant than the wild-type strain of E. coli K-12. In contrast, recBC and recA13 mutants were more sensitive to 5-**azacytidine** than the wild type. Transient exposure of E. coli to 5-**azacytidine** for 60 min induced both recA-dependent inhibition of cell division and induction of lambda prophage in Dcm+ strains but not in Dcm- mutants. Expression of both functions was dependent on recBC exonuclease. On the other hand, 5-**azacytidine** was unable to trigger the induction of umuCD and mucB genes and no amplification of RecA protein synthesis in either Dcm+ or Dcm- strains was observed. These last results are in agreement with previously reported data suggesting that there is a discrimination in the expression of the several SOS functions and that some SOS genes may be induced without amplification of RecA protein synthesis.

L5 ANSWER 18 OF 38 MEDLINE DUPLICATE 10
ACCESSION NUMBER: 85201710 MEDLINE
DOCUMENT NUMBER: 85201710 PubMed ID: 2581700
TITLE: Thymus hormones do not induce proliferative ability or cytolytic function in PNA+ cortical thymocytes.
AUTHOR: Andrews P; Shortman K; Scollay R; Potworowski E F; Kruisbeek A M; Goldstein G; Trainin N; Bach J F
CONTRACT NUMBER: AI 17310 (NIAID)
SOURCE: CELLULAR IMMUNOLOGY, (1985 Apr 1) 91 (2) 455-66.
Journal code: CQ9; 1246405. ISSN: 0008-8749.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198507
ENTRY DATE: Entered STN: 19900320
Last Updated on STN: 19980206
Entered Medline: 19850712

AB A variety of thymus hormone preparations, as well as drugs known to perturb cell differentiation, were tested for their ability to induce nonfunctional cortical thymocytes to become functional precursor cells. Murine cortical thymocytes, defined as the high peanut agglutinin (PNA) binding or as the low H-2K, major [86%] thymocyte subpopulation, were isolated by fluorescence-activated cell sorting. Their function was assessed in a high cloning efficiency, growth factor saturated, concanavalin A-stimulated limit-dilution culture system, determining the number of precursors of extended clones (PTL-p), or determining with a lectin-mediated tumor-**lysis** readout the number of precursors of cytolytic clones (CTL-p). The hormone preparations tested were crude or partially purified culture supernatants from thymus "epithelial" monolayers (TES), soluble extracts of thymic nonlymphoid tissue (STF), semipure thymus humoral factor (THF), and the pure peptides thymopoietin 32-36 (TP5) and "facteur thymique serique" (FTS). These preparations were either added directly to the limit dilution cultures, or were first preincubated with the cells, which were then subjected to limit-dilution culture. In no case did the hormone preparations cause any increase in the level of PTL-p or CTL-p in the PNA+ or low H-2K thymocyte population, even though a conversion of only a few percent to functional cells could have been detected. Two possible explanations are considered. One is that the main function of these materials is to control post-thymic peripheral T cells, rather than to induce intrathymic differentiation. Another is that

the typical cortical thymocyte is beyond the stage at which thymocytes can be induced by hormones, a view that is strengthened by the failure of either 5-**azacytidine** or the phorbol ester 12-O-tetradecanoyl phorbol 13-acetate to activate these cells. In this latter explanation the true intrathymic target of hormone action may be an earlier, and very minor, thymus subpopulation.

L5 ANSWER 19 OF 38 MEDLINE DUPLICATE 11
ACCESSION NUMBER: 85171932 MEDLINE
DOCUMENT NUMBER: 85171932 PubMed ID: 2580434
TITLE: Transfer of human and murine globin-gene sequences into transgenic mice.
AUTHOR: Humphries R K; Berg P; DiPietro J; Bernstein S; Baur A; Nienhuis A W; Anderson W F
SOURCE: AMERICAN JOURNAL OF HUMAN GENETICS, (1985 Mar) 37 (2) 295-310.
Journal code: 3IM; 0370475. ISSN: 0002-9297.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198505
ENTRY DATE: Entered STN: 19900320
Last Updated on STN: 19900320
Entered Medline: 19850513

AB We have studied the transfer of human and murine globin gene sequences into fertilized mouse oocytes by microinjection. Germline transmission was demonstrated for the human delta- and beta-globin genes contained in the **bacteriophage** lambda H beta G1. Expression of these human globin-gene sequences was not detectable in either erythroid or nonerythroid tissues. A recombinant plasmid containing the murine beta maj promoter region coupled to the prokaryotic coding sequence for galactokinase was also successfully transferred to two mice, and stable germline transmission of integrated DNA was demonstrated for at least 3 generations. Despite the presence of a murine globin-promoter sequence, expression of the mouse beta maj galactokinase fusion gene was not observed in primary or secondary animals in erythroid or nonerythroid tissues. Analysis of primary and secondary animals from both series of injections revealed extensive de novo methylation in the integrated microinjected DNA. Administration of 5-**azacytidine** to mice containing the mouse beta maj-promoted galactokinase gene resulted in partial hypomethylation was associated with an apparent two- to threefold increase in galactokinase (gal K) gene expression.

L5 ANSWER 20 OF 38 MEDLINE DUPLICATE 12
ACCESSION NUMBER: 84180376 MEDLINE
DOCUMENT NUMBER: 84180376 PubMed ID: 6201262
TITLE: A new murine model system for the in vitro development of thymoma cell heterogeneity.
AUTHOR: MacLeod C L; Hays E F; Hyman R; Bourgeois S
CONTRACT NUMBER: CA06932 (NCI)
CA12386 (NCI)
CA13287 (NCI)
+
SOURCE: CANCER RESEARCH, (1984 May) 44 (5) 1784-90.
Journal code: CNF; 2984705R. ISSN: 0008-5472.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198406
ENTRY DATE: Entered STN: 19900319
Last Updated on STN: 19970203
Entered Medline: 19840601

AB We have established and characterized a continuous T-cell line derived from the bone marrow of an AKR mouse with disseminated lymphoma. The original tumor cell line is heterogeneous with respect to several markers of thymocyte differentiation. Clones from the line differ in the expression of ThB, Pgp-1, and H-2Kk surface antigens. These clones also differ in their sensitivity to glucocorticoid-induced cell **lysis** . The quantity, affinity, and nuclear translocation properties of the glucocorticoid receptor are similar in the hormone-sensitive and -resistant clones. Furthermore, dexamethasone-resistant T-cells can be selected in vitro from freshly cloned cells sensitive to hormone-induced **lysis** at high frequency and without mutagenesis. Of several randomly sampled, spontaneously arising, independently derived dexamethasone resistant clones, all show a coordinate reduction in cell surface Thy-1 and ThB expression with no detectable changes in

glucocorticoid receptor properties. Following treatment with the DNA-demethylating agent 5-**azacytidine**, the original dexamethasone-resistant T-cell line as well as the dexamethasone-resistant derivatives obtained in vitro regain sensitivity to **lysis**. These results collectively suggest a role of DNA methylation in hormone resistance and are consistent with a model of thymocyte differentiation in which a glucocorticoid-sensitive cell is the progenitor of hormone-resistant T-cells.

L5 ANSWER 21 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1984:507155 CAPLUS
DOCUMENT NUMBER: 101:107155
TITLE: Experimental cultivation of M. leprae and M. lepraemurium
AUTHOR(S): Sula, L.; Matejka, M.; Malkova, J.
CORPORATE SOURCE: Inst. Hyg. Epidemiol., Prague, Czech.
SOURCE: Stud. Pneumol. Phtiseol. Cech. (1984), 44(4), 276-82
CODEN: SPPCAC; ISSN: 0371-2222
DOCUMENT TYPE: Journal
LANGUAGE: Czech
AB The growth of Mycobacterium lepraemurium on Ogawa yolk medium was stimulated by **azacytidine**. Stimulation was apparently caused by inhibition of a temperate **phage** carried by M. lepraemurium. The cultivation of Mycobacterium leprae was attempted but was not successful.

L5 ANSWER 22 OF 38 MEDLINE DUPLICATE 13
ACCESSION NUMBER: 84270490 MEDLINE
DOCUMENT NUMBER: 84270490 PubMed ID: 6205264
TITLE: Effects of 5-azacytosine in DNA on enzymic uracil excision.
AUTHOR: Chao T L; Duker N J
CONTRACT NUMBER: CA-00796 (NCI)
CA-12923 (NCI)
ES-02935 (NIEHS)
SOURCE: MUTATION RESEARCH, (1984 Jun-Jul) 140 (2-3) 93-8.
Journal code: NNA; 0400763. ISSN: 0027-5107.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198408
ENTRY DATE: Entered STN: 19900320
Last Updated on STN: 19970203
Entered Medline: 19840829
AB PBS-2 **phage** DNA, which contains uracil in place of thymine, was used as substrate for both purified B. subtilis uracil-DNA glycosylase and a crude extract from M. luteus. Addition of [3H]5-**azacytidine** to the medium after **phage** infection resulted in substitution of 1.2% azacytosine for cytosine in DNA. Substrate DNA was also labeled with [14C]uracil. Neither enzyme preparation released tritiated bases from DNA. Analysis by S1 nuclease digestion show no increase in single-strandedness of the modified DNA. Enzymic release of uracil by the M. luteus extract was reduced by about 50% from the substituted substrate. By contrast, the rate of uracil excision by the purified enzyme was unaffected by the presence of DNA 5-azacytosine.

L5 ANSWER 23 OF 38 WPIDS COPYRIGHT 2002 DERWENT INFORMATION LTD
ACCESSION NUMBER: 1983-840428 [50] WPIDS
DOC. NO. CPI: C1983-120952
TITLE: Microcapsules with mixed wall formed by interfacial crosslinking - of water soluble poly holoside and protein, esp. for sustained release of pharmaceuticals.
DERWENT CLASS: A96 B07 D13 P73
INVENTOR(S): GOURDIER, B; LEVY, M C
PATENT ASSIGNEE(S): (CNRS) CNRS CENT NAT RECH SCI
COUNTRY COUNT: 13
PATENT INFORMATION:

PATENT NO	KIND	DATE	WEEK	LA	PG
EP 95968	A	19831207	(198350)*	FR	22
R: AT BE CH DE FR GB IT LI LU NL SE					
FR 2527438	A	19831202	(198402)		
JP 58214336	A	19831213	(198404)		
EP 95968	B	19861112	(198646)	FR	
R: AT BE CH DE FR GB IT LI LU NL SE					
DE 3367517	G	19870102	(198701)		
US 4780321	A	19881025	(198845)		
JP 04024091	B	19920424	(199221)		8

APPLICATION DETAILS:

PATENT NO	KIND	APPLICATION	DATE
EP 95968	A	EP 1983-401009	19830520
FR 2527438	A	FR 1982-9156	19820526
JP 58214336	A	JP 1983-93328	19830526
US 4780321	A	US 1986-894383	19860807
JP 04024091	B	JP 1983-93328	19830526

FILING DETAILS:

PATENT NO	KIND		PATENT NO
JP 04024091	B	Based on	JP 58214336

PRIORITY APPLN. INFO: FR 1982-9156 19820526
AN 1983-840428 [50] WPIDS
AB EP 95968 A UPAB: 19930925
Microcapsules have a mixed outer wall obtd. by interfacial copolymerisation of a mixt. of (1) at least one water soluble polyhaloside (I) or its deriv. and (2) at least one protein (II), using a bifunctional, acylating crosslinking agent. Esp. (II) is casein or gelatin and the microcapsules have mean dia. 25-300 microns with wall thickness 1-2 microns.

The microcapsules are made from an aq. alkaline soln. of (I) and (II), of pH over 10, emulsified in an immiscible organic solvent. The stirred emulsion is treated with the crosslinking agent, added as a soln. in organic solvent, and stirred until interfacial crosslinking has occurred. The capsules are recovered by dilution, decantation, centrifuging and washing.

The capsules are pref. formulated to contain pharmaceuticals, esp. for sustained release of alkali salts of carboxylic acids, e.g. salicylic acid. They can also contain food materials or essential oils.
0/2
ABEQ EP 95968 B UPAB: 19930925
Microcapsules containing a pharmaceutically active substance, which microcapsules are resistant to gastric juices and enterosoluble, of the type having an outer wall obtained by the interfacial reticulation of a polyholoside with the aid of a reticulating agent constituted by a bifunctional acylating reagent, characterised in that the composite external wall of the microcapsules is obtained by interfacial reticulation of a mixture comprising, on the one hand, at least one water-soluble polyholoside or water-soluble polyholoside derivative and, on the other hand, at least one protein.
ABEQ US 4780321 A UPAB: 19930925
New process for prepn. of microcapsules having mixed walls contg. drug etc., comprises dissolving mixed water-sol. polyhoside or deriv., protein and active substance in aq. soln., pH 10+, with soln. emulsified by dispersion in immiscible organic solvent by stirring with water in oil type emulsifier. Then reticulation agent dissolved in same organic solvent is stirred into it to give an interfacial reticulum. Microcapsules are sepd. by dilution with same solvent and centrifugation.

Polyhoside may be alkali, cellulose, soln. starch, low M.W. dextrans, hydroxypropylcellulose, gum arabic, gum guar. Solvent may be 1-2C chlorinated hydrocarbon, 5-7C non-chlorinated hydrocarbon. Reticulation agent may be succinyl-, sebaconyl-, or terephthaloyl-dichloride or toluene- or **hexamethylene-diisocyanate**. Vol. reticulation agent solvent is equal to vol. organic solvent in emulsion, to give reticulum conc. change from satd. to 0.1M.

ADVANTAGE - Reticulated microcapsules protect drugs or essential oils from GI.**lysis** or hydrolysis better than those contg. protein alone.

L5 ANSWER 24 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1983:192648 CAPLUS
DOCUMENT NUMBER: 98:192648
TITLE: Role of de novo DNA methylation in the glucocorticoid resistance of a T-lymphoid cell line
AUTHOR(S): Gasson, Judith C.; Ryden, Thomas; Bourgeois, Suzanne
CORPORATE SOURCE: Regulatory Biol. Lab., Salk Inst., San Diego, CA, 92138, USA
SOURCE: Nature (London) (1983), 302(5909), 621-3
CODEN: NATUAS; ISSN: 0028-0836
DOCUMENT TYPE: Journal
LANGUAGE: English
AB The DNA of dexamethasone [50-02-2]-sensitive subclones, obtained by

treatment of the T-lymphoid cell line SAK8 of mouse with 5-**azacytidine** [320-67-2], was spontaneously methylated in cytosine [71-30-7] residues; the spontaneous methylation of DNA was accompanied by an acquisition of the glucocorticoid-resistant phenotype. The revertant cell line, SAK8.A2 became glucocorticoid-sensitive when treated with 5-**azacytidine** and dexamethasone. In SAK8 cell DNA, .apprx.4.5% of the cytosine residues were methylated. After 5-**azacytidine** treatment and subcloning, the 5-methylcytosine [554-01-8] content of the DNA was decreased by nearly 50% (2.5% of total cytosines were methylated). The acquisition of glucocorticoid resistance was accompanied by a gradual increase in 5-methylcytosine content from 2.5 to 3.8%. The **lysis** induced by dexamethasone and 5-**azacytidine** treatment of revertant cells did not result from nonspecific toxicity, as shown with the glucocorticoid receptor mutant cell line W7.

L5 ANSWER 25 OF 38 MEDLINE DUPLICATE 14
ACCESSION NUMBER: 83161274 MEDLINE
DOCUMENT NUMBER: 83161274 PubMed ID: 6601107
TITLE: A new determinant of glucocorticoid sensitivity in lymphoid cell lines.
AUTHOR: Gasson J C; Bourgeois S
CONTRACT NUMBER: 1-F32-AM06179 (NIADDK)
5-R01-GM20868 (NIGMS)
SOURCE: JOURNAL OF CELL BIOLOGY, (1983 Feb) 96 (2) 409-15.
Journal code: HMV; 0375356. ISSN: 0021-9525.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198305
ENTRY DATE: Entered STN: 19900318
Last Updated on STN: 19970203
Entered Medline: 19830505

AB The SAK cell line, derived from a spontaneous thymic lymphoma in an AKR mouse, is resistant to **lysis** by glucocorticoids in spite of the presence of functional glucocorticoid receptor. Receptor function was determined by hormone binding analyses, as well as characterization of hormonal effects on cell growth and on the accumulation of murine leukemia virus and metallothionein mRNAs. SAK cells were fused with a receptor-defective (and therefore resistant) variant of a well-characterized murine thymoma line, W7. The resulting hybrids are glucocorticoid sensitive, demonstrating complementation of the receptor defect in W7 cells by the functional glucocorticoid receptor of SAK. This fusion shows that SAK cells are resistant to the hormone due to the absence of another function designated "I" for **lysis**. SAK cells were also fused with glucocorticoid-sensitive W7 cells (containing wild-type receptor), generating glucocorticoid-sensitive hybrids, which demonstrate that the dexamethasone-resistant phenotype of the SAK cells is recessive. Resistant derivatives of this hybrid were found which still contain the full amount of receptor. Chromosome analysis revealed that, on the average, the resistant derivatives had lost two chromosomes, suggesting segregation of chromosomes carrying genetic material necessary for the "**lysis**" function. The drug 5-**azacytidine** (a known inhibitor of DNA methylation) has been shown to cause heritable changes in gene expression. Treatment of SAK cells with 5-**azacytidine** generated glucocorticoid-sensitive clones at high frequency, suggesting that the gene(s) involved in the "**lysis**" function are intact and have been inactivated through a process such as differentiation.

L5 ANSWER 26 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1983:210221 CAPLUS
DOCUMENT NUMBER: 98:210221
TITLE: Determinants of glucocorticoid resistance in lymphoid cell lines
AUTHOR(S): Gasson, Judith C.; Bourgeois, Suzanne
CORPORATE SOURCE: Regulatory Biol. Lab., Salk Inst., San Diego, CA, 92138, USA
SOURCE: UCLA Symp. Mol. Cell. Biol., New Ser. (1983), 4(Ration. Basis Chemother.), 153-76
CODEN: USMBD6
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Evidence is presented for the existence of 2 bases for glucocorticoid (dexamethasone [50-02-2]) resistance in murine lymphoid T cell lines, namely a receptor defect or a defect in expression of **lysis** function. The receptor defects obsd. in glucocorticoid resistant variants derived from S49 and W7 lines appear to be of genetic origin since their

frequency of appearance is low, dependent on ploidy of r+ allele, and increased by various mutagens. In contrast, the **lysis** defect obsd. in SAK cells appears to be of epigenetic origin since the expression of the **lysis** function is activated at high frequency by treatment with 5-**azacytidine** [320-67-2]. SAK cells show a redn. in cloning efficiency in the presence of glucocorticoids which can be reversed by addn. of culture supernatants contg. growth factors. The clin. relevance of these findings is discussed.

L5 ANSWER 27 OF 38 MEDLINE DUPLICATE 15
ACCESSION NUMBER: 82074941 MEDLINE
DOCUMENT NUMBER: 82074941 PubMed ID: 7309297
TITLE: Induction of target antigens and conversion to susceptible phenotype of NK-cell-resistant lymphoid cell line.
AUTHOR: Clark E A; Sturge J C; Falk L A Jr
CONTRACT NUMBER: CA26713 (NCI)
RR00166 (NCRR)
RR00168 (NCRR)
+
SOURCE: INTERNATIONAL JOURNAL OF CANCER, (1981 Nov 15) 28 (5) 647-54.
Journal code: GQU; 0042124. ISSN: 0020-7136.
PUB. COUNTRY: Denmark
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198202
ENTRY DATE: Entered STN: 19900316
Last Updated on STN: 19970203
Entered Medline: 19820222
AB Two autologous Herpesvirus papio producer lymphoid cell lines and one autologous non-producer line were compared for susceptibility to natural killer (NK) cell-mediated **lysis**. The non-producer cell line, 26CB-1, was more resistant to NK cell killing compared to one viral producer counterpart 13CB-1, but equally resistant when compared to another, 8CB-1. Treatment with chemical agents that affect differentiation or activate the viral cycle, including n-butyrate, IuDR, 5-**azacytidine** and tunicamycin, increased the susceptibility to killing of the non-producer line but had less effect on the 13CB-1 producer line. The increase in susceptibility was due to induction of new target antigens: activated 26CB-1 cells were more effective at inhibiting NK-cell-mediated **lysis** and were bound by more NK cells than untreated control cells. The expression of NK target structures may be related to the differentiated state rather than to the viral production status to target cells.

L5 ANSWER 28 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1975:455604 CAPLUS
DOCUMENT NUMBER: 83:55604
TITLE: Photobiological behavior of bacteria and phages supplemented with aza-analogs of nucleic acid bases
AUTHOR(S): Kittler, L.; Hradecna, Z.; Jacob, H. E.; Loeber, G.
CORPORATE SOURCE: Zentralinst. Mikrobiol. Exp. Ther., DAW, Jena, E. Ger.
SOURCE: Z. Allg. Mikrobiol. (1975), 15(5), 323-31
CODEN: ZAPOAK
DOCUMENT TYPE: Journal
LANGUAGE: English
AB 5-Azacytosine, 5-**azacytidine**, 6-azacytosine, 6-**azacytidine**, and 8-azaadenine were previously shown to be more, and 6-azathymine (I) and 6-azauracil (II) less sensitive to uv light as compared to the corresponding natural nucleic acid bases. The survival curves of Escherichia coli B and K12 C600 and Bacillus cereus supplemented with any of the analogs except I and II showed an increased uv sensitivity, whereas previous studies showed a decreased sensitivity of Streptococcus faecalis cultures supplemented with I (H. L. Guenther and W. H. Prusoff, 1967). The lack of any significant influence on inactivation curves of E. coli K12 C600 by I and II, or of E. coli phages .lambda. cb2 and .lambda. c2b5 by 5-**azacytidine** is discussed in terms of too low an incorporation rate into nucleic acids in vivo. No discrimination was made with respect to DNA vs. RNA incorporation.

L5 ANSWER 29 OF 38 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.
ACCESSION NUMBER: 74045357 EMBASE
DOCUMENT NUMBER: 1974045357
TITLE: Bronchogenic carcinoma: New drugs available for study.
AUTHOR: Slavik M.; Carter S.K.
CORPORATE SOURCE: Div. Cancer Treatm., Cancer Ther. Evaluat. Branch, Nat. Cancer Inst., Bethesda, Md., United States

SOURCE: CANCER CHEMOTHER.REP., (1973) 4/2 (III) (265-269).
CODEN: CNCRA6
DOCUMENT TYPE: Journal
FILE SEGMENT: 038 Adverse Reactions Titles
037 Drug Literature Index
016 Cancer
015 Chest Diseases, Thoracic Surgery and Tuberculosis
030 Pharmacology
LANGUAGE: English
AB Bronchial carcinoma is considered one of the 'signal' tumor types in which new antineoplastic drugs sponsored by the Division of Cancer Treatment, National Cancer Institute, are tested. Among the new drugs which have completed phase I evaluation and are ready for **phage** II study in bronchial carcinoma are cis diamminedichloroplatinum, isophosphamide, and 5 **azacytidine**. Chromomycin A3 has entered phase I studies. Preclinical and clinical data on these drugs with respect to bronchial carcinoma are discussed.

L5 ANSWER 30 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1972:537285 CAPLUS
DOCUMENT NUMBER: 77:137285
TITLE: Components of the nucleoside-transporting system in Escherichia coli
AUTHOR(S): Doskocil, J.
CORPORATE SOURCE: Inst. Org. Chem. Biochem., Czech. Acad. Sci., Prague, Czech.
SOURCE: Biochim. Biophys. Acta (1972), 282(1), 393-400
CODEN: BBACAQ
DOCUMENT TYPE: Journal
LANGUAGE: English
AB A mutant of E. coli resistant to 5-**azacytidine** was shown to be deficient in the high-affinity component of the nucleoside-transporting system which participates in several types of conversion of nucleosides, such as incorporation and deamination of 5-**azacytidine** and cytidine as well as phosphorolysis of thymidine. All these reactions are affected in a similar manner when wild-type cells are infected with T4-**phage** or treated with osmotically disrupted **phage**, while the treatment of mutant bacteria with this **phage** does not significantly alter the value of the Michaelis const. for the phosphorolysis of thymidine. Since the metabolic conversion of nucleosides in mutant cells remains susceptible to competitive inhibition with heterologous nucleosides, it seems that another, low-affinity component participates in the transport, which is still active in mutant or **phage**-infected bacteria. Cytidine is transported predominantly by the low-affinity component, provided its concn. in the medium is sufficiently high; 5-**azacytidine**, requires the high-affinity component for efficient transport at any concn.

L5 ANSWER 31 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1972:81626 CAPLUS
DOCUMENT NUMBER: 76:81626
TITLE: Differential incorporation of 5-azapyrimidines into the RNA of **phage** f2 and of bacterial host
AUTHOR(S): Doskocil, Jiri; Sorm, Frantisek
CORPORATE SOURCE: Ustav Org. Chem. Biochem., Cesk. Akad. Ved, Prague, Czech.
SOURCE: Eur. J. Biochem. (1971), 23(2), 253-61
CODEN: EJBCAI
DOCUMENT TYPE: Journal
LANGUAGE: English
AB 5-Azacytosine [931-86-2] was incorporated in place of cytosine [71-30-7], and to a lesser extent in place of uracil [66-22-8] in **phage** f2-infected Escherichia coli K12 cultures. Particles of **phage** f2 formed in the presence of 5-**azacytidine** had an almost normal sedimentation coefficient and buoyant density, but their infectivity with respect to populations of normal **phage** was reduced. RNA extracted from these particles was partly degraded to small fragments and had very low template activity. **Phage** RNA replicase utilized both 5-**azacytidine** triphosphate [2226-74-6] and 5-azauridine triphosphate [34330-32-0] in vivo for the synthesis of RNA. **Phage** RNA, however, contained less 5-azacytosine than either replicative-form RNA or host RNA.

L5 ANSWER 32 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1970:453256 CAPLUS
DOCUMENT NUMBER: 73:53256
TITLE: Inhibitory effects of 5-**azacytidine** and 5-azauridine in Escherichia coli

AUTHOR(S): Doskocil, Jiri; Sorm, Frantisek
CORPORATE SOURCE: Cesk. Akad. Ved, Prague, Czech.
SOURCE: Collect. Czech. Chem. Commun. (1970), 35(6), 1880-91
CODEN: CCCCAK
DOCUMENT TYPE: Journal
LANGUAGE: English

AB 5-**Azacytidine** (I) and 5-azauridine (II) caused complete arrest of growth and total protein synthesis, measured by incorporation of leucine, in wild-type strains of E. coli. In cytidine deaminase-deficient strains, the inhibition was equally effective after II but only mild after I. I inhibited the synthesis of .beta.-galactosidase under conditions excluding inhibition of total protein synthesis. I and II inhibited the replication of T4 **phage** DNA, if added simultaneously with the infecting **phage**, but I showed a stronger effect than II when added 8 min later. Addn. of II to a culture infected with **phage** T4 stopped the incorporation of leucine 4-5 min later, while in a wild-type T4-infected culture leucine incorporation decreased slightly on addn. of I 9-14 min after the infection. In deaminase-deficient T4-infected culture, I affected the leucine incorporation very slightly, whereas the replication of **phage** DNA was inhibited as markedly as in wild-type T4-infected cells. II formed by deamination of I apparently is responsible for the inhibition of total protein synthesis, while I primarily interferes with the replication of T4 **phage** DNA. The relative insensitivity of protein synthesis in T4-infected cells to the effect of I in comparison with uninfected host is explained by a strong depression of deamination after infection with T4 **phage**.

L5 ANSWER 33 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1970:129598 CAPLUS
DOCUMENT NUMBER: 72:129598
TITLE: Mode of action of 5-aza-2'-deoxycytidine in Escherichia coli
AUTHOR(S): Doskocil, Jiri; Sorm, Frantisek
CORPORATE SOURCE: Ustav Org Chem. Biochem., Cesk. Akad. Ved., Prague, Czech.
SOURCE: Eur. J. Biochem. (1970), 13(1), 180-7
CODEN: EJBCAI
DOCUMENT TYPE: Journal
LANGUAGE: English

AB 5-Azadeoxycytidine is a good donor of deoxyribosyl group in reactions catalyzed by pyrimidine deoxyriboside phosphorylase, promoting the deoxyriboside-dependent incorporation of thymine by wild-type cells of E. coli about twice as effectively as either deoxycytidine or deoxyuridine. Prior deamination to 5-azadeoxyuridine is necessary for the utilization of the deoxyribose moiety of 5-azadeoxycytidine, since the deoxyribosyl-donor activity is entirely lost in mutants deficient in cytidine deaminase. Upon infection of wild-type cells with **phage** T4 the deamination of 5-azac ytidine and 5-azadeoxycytidine is strongly depressed; a proportional los s of the deoxyribosyl-donor ability of the latter is obsd. while the activity of deoxycytidine remains unaffected. In wild-type cells of E. coli 5-azadeoxycytidine inhibits protein synthesis and the replication of **phage** f2 as effectively as 5-**azacytidine**, but has much less effect on the replication of **phage** T4. In deaminase-less mutants 5-azadeoxycytidine has practically no inhibitory effects. It is concluded that 5-azadeoxycytidine enters the cells of E. coli via deamination, followed by phosphorolytic cleavage of the glycosidic bond. Direct utilization of the deoxynucleoside, conserving the glycosidic bond and the 6-amino group, does not occur to any appreciable extent. In accord with this finding the biol. effects of 5-azadeoxycytidine are si milar to those of 5-azauridine but different from the action of 5-**azacytidine**.

L5 ANSWER 34 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1970:97628 CAPLUS
DOCUMENT NUMBER: 72:97628
TITLE: Effects of 5-**azacytidine** and 5-azauridine on protein synthesis in Escherichia coli
AUTHOR(S): Doskocil, Jiri; Sorm, Frantisek
CORPORATE SOURCE: Inst. Org. Chem. Biochem., Cesk. Akad. Ved, Prague, Czech.
SOURCE: Biochem. Biophys. Res. Commun. (1969), 38(4), 569-74
CODEN: BBRCA9
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Wild-type E. coli has strong cytidine deaminase activity which rapidly deaminates 5-**azacytidine** into 5-azauridine, thus making it difficult to resolve the biol. activity of these compds. Therefore, E. coli mutants deficient in cytidine deaminase activity were used to resolve

the biol. activity of 5-**azacytidine**. 5-**Azacytidine** only weakly inhibited total protein synthesis in such mutants, in spite of being extensively incorporated into RNA. Marked inhibition of protein synthesis in wild-type strains was due to the formation of 5-azauridine from 5-**azacytidine**. However, the blocking of replication of **phage** T4, which is due primarily to inhibition of replication of **phage** DNA, was a function of 5-**azacytidine** itself.

L5 ANSWER 35 OF 38 MEDLINE DUPLICATE 16
ACCESSION NUMBER: 69169541 MEDLINE
DOCUMENT NUMBER: 69169541 PubMed ID: 4889173
TITLE: Incorporation and phosphorylation of 5-**azacytidine** by normal and T4-**phage**-infected cells of E. coli.
AUTHOR: Doskocil J; Sorm F
SOURCE: EUROPEAN JOURNAL OF BIOCHEMISTRY, (1969 Mar) 8 (1) 75-80. Journal code: EMZ; 0107600. ISSN: 0014-2956.
PUB. COUNTRY: GERMANY, WEST: Germany, Federal Republic of
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 196906
ENTRY DATE: Entered STN: 19900101
Last Updated on STN: 19900101
Entered Medline: 19690609

L5 ANSWER 36 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1969:26620 CAPLUS
DOCUMENT NUMBER: 70:26620
TITLE: Effect of azapyrimidine nucleosides and deoxynucleosides on the metabolism of thymidine and thymine in cultures of Escherichia coli
AUTHOR(S): Doskocil, Jiri; Paces, V.
CORPORATE SOURCE: Ceskoslov. Akad. Ved, Prague, Czech.
SOURCE: Collect. Czech. Chem. Commun. (1968), 33(12), 4369-78
CODEN: CCCCAK
DOCUMENT TYPE: Journal
LANGUAGE: English
AB 5-**Azacytidine** (I) and deoxy-5-**azacytidine** (II) inhibit phosphorolysis of thymidine by normal cells of E. coli and by bacteria infected with T4 **phage**. The inhibitory effect is similar to that of the ribo- and deoxyribonucleosides of cytosine, uracil, and adenine, whereas deoxy-6-azauridine (III), deoxy-6-**azacytidine** (IV), deoxy-5-hydroxymethyl-6-**azacytidine** (V), guanosine, and deoxyguanosine are without effect. There is no inhibition of phosphorolysis in cell-free exts. II is a good deoxyribosyl donor for normal cells in the deoxyriboside-dependent incorporation of thymine, but has a low donor activity in cells infected with **phage** TV, while III, IV, and V are without activity at all. They also fail to affect the production of viable **phage**, whereas I and II have a marked inhibitory effect. Inhibition of phosphorolysis is probably not due to direct interaction of nucleosides with thymidine phosphorylase. Specific binding sites probably exist on the surface of bacterial cells where thymidine is bound before phosphorolysis can take place.

L5 ANSWER 37 OF 38 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1968:57567 CAPLUS
DOCUMENT NUMBER: 68:57567
TITLE: Effect of nucleosides on the phosphorolysis of thymidine by normal and **phage**-infected cells of Escherichia coli
AUTHOR(S): Doskocil, Jiri; Paces, V.
CORPORATE SOURCE: Ceskoslov. Akad. Ved, Prague, Czech.
SOURCE: Biochem. Biophys. Res. Commun. (1968), 30(2), 153-8
CODEN: BBRCA9
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Addn. of pyrimidine nucleosides and deoxynucleosides, deoxyadenosine, adenosine, and 5-**azacytidine**, but not deoxyguanosine, 6-**azacytidine**, or 6-azauridine, inhibited phosphorolysis of thymidine in uninfected or T4 **phage**-infected cultures of E. coli. In infected cells, the added nucleosides and deoxynucleosides had only slight effects on the rate of incorporation of thymidine. In uninfected cells, deoxyadenosine increased the rate of incorporation of thymidine by 50%, while deoxycytidine reduced this rate .apprx.2-fold. The ribonucleosides prevented the phosphorolysis of thymidine, but were without effect on the rate of its incorporation. The inhibition of phosphorolysis by nucleosides and deoxynucleosides was observed only with intact cells. The specificity pattern of the inhibitory action of the

compds. on thymidine phosphorolysis in vivo was entirely different from that of substrates and inducers of thymidine phosphorylase, indicating that direct interaction of the nucleosides with the enzyme is probably not the cause of the inhibition.

L5 ANSWER 38 OF 38 MEDLINE DUPLICATE 17
ACCESSION NUMBER: 68088332 MEDLINE
DOCUMENT NUMBER: 68088332 PubMed ID: 4863910
TITLE: The action of 5-**azacytidine** on bacteria infected with **bacteriophage** T4.
AUTHOR: Doskocil J; Sorm F
SOURCE: BIOCHIMICA ET BIOPHYSICA ACTA, (1967) 145 (3) 780-91.
Journal code: A0W; 0217513. ISSN: 0006-3002.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 196802
ENTRY DATE: Entered STN: 19900101
Last Updated on STN: 19900101
Entered Medline: 19680213

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	4	"1439121"	USPAT; EPO; JPO; DERWENT	2002/03/05 10:14
2	L6	15346	azacytidine or (hexamethylene adj diisocyanate)	USPAT; EPO; JPO; DERWENT	2002/03/05 10:15
3	L11	60	l6 and (bacteriophage? or phage or phagolysis)	USPAT; EPO; JPO; DERWENT	2002/03/05 10:16
4	L16	16	l11 and lacto$	USPAT; EPO; JPO; DERWENT	2002/03/05 10:18

09/720,0960 Search Strategy/Results

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     (FILE 'HOME' ENTERED AT 07:05:00 ON 05 MAR 2002)

     FILE 'MEDLINE, AGRICOLA, CAPLUS, BIOSIS, EMBASE, WPIDS' ENTERED AT
     07:05:22 ON 05 MAR 2002
L1             1 S DN209

     FILE 'CAPLUS' ENTERED AT 07:06:14 ON 05 MAR 2002
                E NILSSON D/AU 25
L2             1 S (E3 OR E6 OR E7 OR E8) AND (AUXOTROPH)
L3            82 S (PURINE OR THYMIDINE) (W) (AUXOTROPH OR AUXOTROPHIC)
L4             3 S L3 AND LACTO?
L5             0 S L3 AND (LACTIC (W) ACID)

=>
```

L2 ANSWER 1 OF 1 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1995:482884 CAPLUS
DOCUMENT NUMBER: 123:75905
TITLE: Isolation of Lactococcus lactis nonsense suppressors and construction of a food-grade cloning vector
AUTHOR(S): Dickely, Francoise; **Nilsson, Dan**; Hansen, Egon Bech; Johansen, Eric
CORPORATE SOURCE: Dep. Genet., Christian Hansen's Laboratorium Danmark A/S, Horsholm, DK-2970, Den.
SOURCE: Mol. Microbiol. (1995), 15(5), 839-47
CODEN: MOMIEE; ISSN: 0950-382X
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Nonsense suppressor strains of Lactococcus lactis were isolated using plasmids contg. nonsense mutations or as revertants of a nonsense auxotrophic mutant. The nonsense suppressor gene was cloned from two suppressor strains and the DNA sequence detd. One suppressor is an ochre suppressor with an altered tRNAgin and the other an amber suppressor with an altered tRNAser. The nonsense suppressors allowed isolation of nonsense mutants of a lytic bacteriophage and suppressible auxotrophic mutants of L. lactis MG1363. A food-grade cloning vector based totally on DNA from Lactococcus and a synthetic poly-linker with 11 unique restriction sites was constructed using the ochre suppressor as a selectable marker. Selection, following electroporation of a suppressible purine **auxotroph**, can be done on purine-free medium. The pepN gene from L. lactis Wg2 was sub-cloned resulting in a good-grade plasmid giving a four- to fivefold increase in lysine aminopeptidase activity.

```
=> s (purine or thymidine) (w) (auxotroph or auxotrophic)
         30994 PURINE
         46709 THYMIDINE
          1931 AUXOTROPH
          3217 AUXOTROPHIC
L3          82 (PURINE OR THYMIDINE) (W) (AUXOTROPH OR AUXOTROPHIC)

=> s l3 and lacto?
        140116 LACTO?
L4           3 L3 AND LACTO?

=> d 1- ibib abs
YOU HAVE REQUESTED DATA FROM 3 ANSWERS - CONTINUE? Y/(N):y
```

L4 ANSWER 1 OF 3 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 2000:259079 CAPLUS
DOCUMENT NUMBER: 133:40345
TITLE: Disruption of cytoplasmic and mitochondrial folylpolyglutamate synthetase activity in Saccharomyces cerevisiae
AUTHOR(S): DeSouza, Leroi; Shen, Yang; Bognar, Andrew L.
CORPORATE SOURCE: Department of Medical Genetics and Microbiology, University of Toronto, Toronto, ON, M5S 1A8, Can.
SOURCE: Arch. Biochem. Biophys. (2000), 376(2), 299-312
CODEN: ABBIA4; ISSN: 0003-9861
PUBLISHER: Academic Press
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Similar to other eukaryotes, yeasts have parallel pathways of C1 metab. in the cytoplasm and mitochondria and have folylpolyglutamate synthetase activity in both compartments. The gene encoding folylpolyglutamate synthetase is MET7 (also referred to as MET23) on chromosome XV and appears to encode both the cytoplasmic and mitochondrial forms of the enzyme. In order to det. the metabolic roles of both forms of folylpolyglutamate synthetase, the met7 gene was disrupted and it was detd. that the strain is a methionine auxotroph and an adenine and **thymidine auxotroph** when grown in the presence of sulfanilamide. The met7 mutant becomes petite under normal growth conditions but can be maintained with a grande phenotype if the strain is tup and all media are supplemented with dTMP. A met7 gly1 strain is auxotrophic for glycine when grown on glucose but prototrophic when grown on glycerol. A met7 ser1 strain cannot use glycine to suppress the serine auxotrophy of the ser1 phenotype. A met7 shm2 strain is nonviable. In order to disrupt just the mitochondrial folylpolyglutamate synthetase activity, mutants with an inactivated chromosomal MET7 gene complemented by genes that express only cytoplasmic folylpolyglutamate synthetase, including the **Lactobacillus** casei folC gene and the yeast MET7

gene with its mitochondrial leader sequence deleted (MET7.DELTA.m), were constructed. All the genes providing cytoplasmic folylpolyglutamate synthetase complemented the methionine auxotrophy as well as the synthetic lethality of the shm2 strain and the synthetic glycine auxotrophy of the gly1 strain. The strains lacking the mitochondrial folylpolyglutamate synthetase had longer doubling times than the isogenic wild-type strains but retained the function of the mitochondrial folate-dependent enzymes to produce formate, serine, and glycine. Mutants complemented by the bacterial folC gene or by the MET7.DELTA.m gene on a 2.mu. plasmid remained grande without the tup mutation and supplementation and dTMP. Mutants complemented by the MET7.DELTA.m gene integrated in single copy became petites under those conditions, indicating a deficiency in dTMP prodn. but this is likely due to lower expression of cytoplasmic folylpolyglutamate synthetase by the MET7.DELTA.m gene. (c) 2000 Academic Press.

REFERENCE COUNT: 35 THERE ARE 35 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L4 ANSWER 2 OF 3 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1995:482884 CAPLUS
DOCUMENT NUMBER: 123:75905
TITLE: Isolation of **Lactococcus** lactis nonsense suppressors and construction of a food-grade cloning vector
AUTHOR(S): Dickely, Francoise; Nilsson, Dan; Hansen, Egon Bech; Johansen, Eric
CORPORATE SOURCE: Dep. Genet., Christian Hansen's Laboratorium Danmark A/S, Horsholm, DK-2970, Den.
SOURCE: Mol. Microbiol. (1995), 15(5), 839-47
CODEN: MOMIEE; ISSN: 0950-382X
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Nonsense suppressor strains of **Lactococcus** lactis were isolated using plasmids contg. nonsense mutations or as revertants of a nonsense auxotrophic mutant. The nonsense suppressor gene was cloned from two suppressor strains and the DNA sequence detd. One suppressor is an ochre suppressor with an altered tRNAgin and the other an amber suppressor with an altered tRNAser. The nonsense suppressors allowed isolation of nonsense mutants of a lytic bacteriophage and suppressible auxotrophic mutants of L. lactis MG1363. A food-grade cloning vector based totally on DNA from **Lactococcus** and a synthetic poly-linker with 11 unique restriction sites was constructed using the ochre suppressor as a selectable marker. Selection, following electroporation of a suppressible **purine auxotroph**, can be done on purine-free medium.
The pepN gene from L. lactis Wg2 was sub-cloned resulting in a good-grade plasmid giving a four- to fivefold increase in lysine aminopeptidase activity.

L4 ANSWER 3 OF 3 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1993:402225 CAPLUS
DOCUMENT NUMBER: 119:2225
TITLE: Isolation of **purine auxotrophic** mutants of **Lactococcus** lactis and characterization of the gene hpt encoding hypoxanthine guanine phosphoribosyltransferase
AUTHOR(S): Nilsson, Dan; Lauridsen, Anette Ager
CORPORATE SOURCE: Dep. Genet., Hansen's Lab. Denmark A/S, Horsholm, DK-2970, Den.
SOURCE: Mol. Gen. Genet. (1992), 235(2-3), 359-64
CODEN: MGGEAE; ISSN: 0026-8925
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Five **purine auxotrophic** mutants of L. lactis were isolated. L. lactis was capable of converting adenine, guanine and hypoxanthine to AMP, GMP and IMP, resp., indicating the existence of adenine phosphoribosyltransferase (APRT) and hypoxanthine guanine phosphoribosyltransferase (HGPRT) activities. A 1.3 kb DNA fragment from L. lactis was cloned by complementation of the hpt mutation in Escherichia coli. Introduction of this fragment into L. lactis resulted in an increase in HGPRT activity. In vitro transcription and translation anal. showed that the fragment coded for a polypeptide with a Mr of 22,000. The nucleotide sequence of this hpt gene was detd.

L1 ANSWER 1 OF 1 WPIDS COPYRIGHT 2002 DERWENT INFORMATION LTD
ACCESSION NUMBER: 2000-013263 [01] WPIDS
DOC. NO. CPI: C2000-002534
TITLE: New vector for lactic acid bacteria starter cultures useful for food products.
DERWENT CLASS: D13 D16
INVENTOR(S): JOHANSEN, E; LARSEN, R; SORENSEN, K I
PATENT ASSIGNEE(S): (CHRH-N) CHR HANSEN AS
COUNTRY COUNT: 87
PATENT INFORMATION:

PATENT NO	KIND	DATE	WEEK	LA	PG
WO 9954488	A1	19991028	(200001)*	EN	61
RW: AT BE CH CY DE DK EA ES FI FR GB GH GM GR IE IT KE LS LU MC MW NL OA PT SD SE SL SZ UG ZW					
W: AE AL AM AT AU AZ BA BB BG BR BY CA CH CN CU CZ DE DK EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT UA UG US UZ VN YU ZA ZW					
AU 9931377	A	19991108	(200014)		
BR 9910132	A	20010109	(200106)		
EP 1073757	A1	20010207	(200109)	EN	
R: AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE					

APPLICATION DETAILS:

PATENT NO	KIND	APPLICATION	DATE
WO 9954488	A1	WO 1999-DK209	19990414
AU 9931377	A	AU 1999-31377	19990414
BR 9910132	A	BR 1999-10132	19990414
		WO 1999-DK209	19990414
EP 1073757	A1	EP 1999-913119	19990414
		WO 1999-DK209	19990414

FILING DETAILS:

PATENT NO	KIND		PATENT NO
AU 9931377	A	Based on	WO 9954488
BR 9910132	A	Based on	WO 9954488
EP 1073757	A1	Based on	WO 9954488

PRIORITY APPLN. INFO: US 1998-82555P 19980421; DK 1998-551 19980421
AN 2000-013263 [01] WPIDS
AB WO 9954488 A UPAB: 20000105
NOVELTY - Recombinant food-grade vector (I) comprising a nonsense suppressor gene encoding a tRNA comprising an amber suppressor, which does not adversely affect the growth and metabolic activity of host strains, is new.

DETAILED DESCRIPTION - (I) consists of lactic acid bacterial DNA, and comprises a replicon which allows (I) to replicate in a lactic acid bacterium. (I) has at least one of the following characteristics:

(i) when present in Lactococcus lactis strain FA4-1-1 (DSM 12086) with an amber mutation in the pyrF gene that is suppressible by the suppressor, it permits growth at 30 deg. C with a doubling time of 100 minutes (maximum) in non-pyrimidine containing minimal medium;

(ii) when present in Lactococcus lactis FH CY-1 with an amber mutation in the pyrF gene (strain CHCC4146, DSM 12109) that is suppressible by the suppressor, it permits the strain to acidify milk at the same rate as the parent strain (FH CY-1, DSM 12087);

(iii) it permits Lactococcus lactis FA4-1-1 to grow at 30 deg. C in non-pyrimidine containing minimal medium with a doubling time less than that for Lactococcus lactis strain **DN209** transformed with vector pFG1.1 (DSM 12088) which contains a gene coding for a suppressor capable of suppressing the amber mutation in the **DN209** strain.

INDEPENDENT CLAIMS are also included for the following:

(1) a lactic acid bacterium (II) comprising (I);

(2) an isolated pure culture of (II);

(3) a composition comprising the culture and a carrier; and

(4) a method of stably maintaining (I) in lactic acid bacterial host cells, comprising transforming the vector into nonsense mutant cells unable to grow without the vector.

USE - The new vector is useful in lactic acid bacterial host cells growing in milk, vegetable materials, meat products, fruit juice, wine,

batter or dough, and is useful in compositions useful as starter cultures in the preparation of dairy flavors e.g. flavoring of butter, margarine, or cheese flavoring products, food and feed products (claimed).

The composition is useful starter cultures used for preservation of animal feeds, and as probiotics.

ADVANTAGE - The new vector does not comprise a gene coding for antibiotic resistance, therefore is food-grade. It does not cause growth inhibition of host cells, and permits the host to acidify milk at the same rate as the same strains without the vector.

Dwg.0/10

09/720,096

~~09/715,479~~ Search Strategy/Results

(FILE 'HOME' ENTERED AT 17:40:05 ON 13 MAR 2002)

FILE 'MEDLINE, AGRICOLA, CAPLUS, BIOSIS, EMBASE, WPIDS' ENTERED AT 17:40:28 ON 13 MAR 2002

L1 5001 S AUXOTROPH
L2 26 S L1 AND (LACTOCOCCUS OR (LACTIC (W) ACID))
L3 11 DUP REM L2 (15 DUPLICATES REMOVED)

FILE 'STNGUIDE' ENTERED AT 17:44:22 ON 13 MAR 2002

L4 0 S AUXOTROPH AND (PHAGE OR BACTERIOPHAGE)

FILE 'MEDLINE, AGRICOLA, CAPLUS, BIOSIS, EMBASE, WPIDS' ENTERED AT 18:05:00 ON 13 MAR 2002

L5 212 S AUXOTROPH AND (PHAGE OR BACTERIOPHAGE)
L6 96 S AUXOTROPH/AB AND (PHAGE OR BACTERIOPHAGE)/AB
L7 0 S L6 AND (FERMENT OR ACIDIFY OR MILK OR CHEESE)
L8 11 S L6 AND ((PURINE (W) AUXOTROPH) OR (THYMIDINE (W) AUXOTROPH)
L9 6 DUP REM L8 (5 DUPLICATES REMOVED)

=>

L3 ANSWER 1 OF 11 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 2001:763228 CAPLUS
DOCUMENT NUMBER: 135:314428
TITLE: Positive selection of transformants by **auxotroph** complementation with enzymatic precursor conversion
INVENTOR(S): Silva, Christopher J.
PATENT ASSIGNEE(S): Cubist Pharmaceuticals, Inc., USA
SOURCE: PCT Int. Appl., 51 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2001077366	A1	20011018	WO 2001-US11567	20010410

W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

PRIORITY APPLN. INFO.: US 2000-195911 P 20000410
AB This invention relates to a pos. selection method, compds. useful for the pos. selection and appropriate hosts. The method permits one to select a host, or **auxotroph**, which may be a prokaryote or an eukaryote, based on the ability of the host to express an enzyme(s) capable of catalyzing a reaction that converts a precursor mol. into a mol. or factor necessary for the host's survival. This invention encompasses methods useful to find new enzymes expressing a desired activity, methods of selecting host cells, methods of maintaining a plasmid within a host that do not utilize antibiotics, and methods of expressing proteins or other materials for com. prodn. purposes.
REFERENCE COUNT: 5 THERE ARE 5 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L3 ANSWER 2 OF 11 EMBASE COPYRIGHT 2002 ELSEVIER SCI. B.V.
ACCESSION NUMBER: 2000410952 EMBASE
TITLE: Physiological evidence for differently regulated tryptophan-dependent pathways for indole-3-acetic acid synthesis in Azospirillum brasilense.
AUTHOR: Carreno-Lopez R.; Campos-Reales N.; Elmerich C.; Baca B.E.
CORPORATE SOURCE: C. Elmerich, Unite de Physiologie Cellulaire, Departement des Biotechnologies, Institut Pasteur, 25-28, Rue du Dr. Roux, 75724 Paris Cedex, France. elmerich@pasteur.fr
SOURCE: Molecular and General Genetics, (2000) 264/4 (521-530).
Refs: 39
ISSN: 0026-8925 CODEN: MGGEAE
COUNTRY: Germany
DOCUMENT TYPE: Journal; Article
FILE SEGMENT: 004 Microbiology
LANGUAGE: English
SUMMARY LANGUAGE: English
AB Disruption of ipdC, a gene involved in indole-3-acetic acid (IAA) production by the indole pyruvate pathway in Azospirillum brasilense Sp7, resulted in a mutant strain that was not impaired in IAA production with lactate or pyruvate as the carbon source. A tryptophan **auxotroph** that is unable to convert indole to tryptophan produced IAA if tryptophan was present but did not synthesise IAA from indole. Similar results were obtained for a mutant strain with additional mutations in the genes ipdC and trpD. This suggests the existence of an alternative Trp-dependent route for IAA synthesis. On gluconate as a carbon source, IAA production by the ipdC mutant was inhibited, suggesting that the alternative route is regulated by catabolite repression. Using permeabilised cells we observed the enzymatic conversion of tryptamine and indole-3-acetonitrile to IAA, both in the wild-type and in the ipdC mutant. IAA production from tryptamine was strongly decreased when gluconate was the carbon source.

L3 ANSWER 3 OF 11 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1997:295334 BIOSIS
DOCUMENT NUMBER: PREV199799594537
TITLE: Cloning and functional expression in Escherichia coli of

the gene encoding the di- and tripeptide transport protein of Lactobacillus helveticus.
AUTHOR(S): Nakajima, Hajime (1); Hagting, Anja; Kunji, Edmund R. S.; Poolman, Bert; Konings, Wil N.
CORPORATE SOURCE: (1) Technol. Res. Inst., Snow Brand Milk Products Co. Ltd., 1-1-2 Minamidai, Kawagoe, 3501-11 Japan
SOURCE: Applied and Environmental Microbiology, (1997) Vol. 63, No. 61, pp. 2213-2217.
ISSN: 0099-2240.
DOCUMENT TYPE: Article
LANGUAGE: English
AB The gene encoding the di- and tripeptide transport protein (DtpT) of Lactobacillus helveticus (DtpT-LH) was cloned with the aid of the inverse PCR technique and used to complement the dipeptide transport-deficient and proline-auxotrophic Escherichia coli E1772. Functional expression of the peptide transporter was shown by the uptake of prolyl-(14C)alanine in whole cells and membrane vesicles. Peptide transport via DtpT in membrane vesicles is driven by the proton motive force. The system has specificity for di- and tripeptides but not for amino acids or tetrapeptides. The dtpT-LH gene consists of 1,491 bp, which translates into a 497-amino-acid polypeptide. DtpT-LH shows 34% identity to the di- and tripeptide transport protein of **Lactococcus** lactis and is also homologous to various peptide transporters of eukaryotic origin, but the similarity between these proteins is confined mainly to the N-terminal halves.

L3 ANSWER 4 OF 11 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1996:300052 CAPLUS
DOCUMENT NUMBER: 124:337531
TITLE: Nutritional requirements of **lactic acid** bacteria: interactions between carbon and nitrogen flux
AUTHOR(S): Loubiere, P.; Novak, L.; Cocaign-Bousquet, M.; Lindley, N. D.
CORPORATE SOURCE: Centre de bio-ingenierie Gilbert-Druand, Institut national des sciences appliquees complexe scientifique de Rangueil, Toulouse, Fr.
SOURCE: Lait (1996), 76(1-2), 5-12
CODEN: LAITAG; ISSN: 0023-7302
DOCUMENT TYPE: Journal
LANGUAGE: French
AB Nutritional requirements have been detd. for two strains of **Lactococcus** lactis subsp. lactis. The IL 1403 dairy strain, was shown to be **auxotroph** for nine amino acids and five vitamins, while the NCDO 2118 strain isolated from vegetable matter was prototroph for all amino acids when using the single omission technique, but nevertheless required six amino acids and five vitamins for growth in minimal medium. In such a medium, the suppression of glutamate led to a lag phase of about 2 days and a decrease of the growth rate. The problem of glutamate synthesis is linked to the part of Krebs cycle between oxaloacetate and .alpha.-ketoglutarate, and to glutamate dehydrogenase. Concerning the branched-chain amino acids, not noly has the regulation of acetolactate decarboxylase been confirmed, but the regulation of acetohydroxy acid synthase(s) by threonine and thiamine has also been obsd. Both the consumption and the synthesis of serine are regulated by other amino acids or vitamins. Serine consumption is about 12 times higher in the minimal medium than in a medium contg. 18 aminoacids. The study of 14C-serine distribution showed that it is deaminated and transformed to lactate.

L3 ANSWER 5 OF 11 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 96074317 MEDLINE
DOCUMENT NUMBER: 96074317 PubMed ID: 7592480
TITLE: Generation of auxotrophic mutants of Enterococcus faecalis.
AUTHOR: Li X; Weinstock G M; Murray B E
CORPORATE SOURCE: Department of Microbiology and Molecular Genetics, University of Texas Medical School, Houston 77030, USA.
CONTRACT NUMBER: R01 AI33516 (NIAID)
SOURCE: JOURNAL OF BACTERIOLOGY, (1995 Dec) 177 (23) 6866-73.
Journal code: HH3; 2985120R. ISSN: 0021-9193.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
OTHER SOURCE: GENBANK-U24682; GENBANK-U24692; GENBANK-U25090; GENBANK-U25091; GENBANK-U25092; GENBANK-U25093; GENBANK-U25094; GENBANK-U25095; GENBANK-U36195
ENTRY MONTH: 199512
ENTRY DATE: Entered STN: 19960124

Last Updated on STN: 19960124
Entered Medline: 19951226

AB A 22-kb segment of chromosomal DNA from Enterococcus faecalis OG1RF containing the pyrimidine biosynthesis genes pyrC and pyrD was previously detected as complementing Escherichia coli pyrC and pyrD mutations. In the present study, it was found that the E. faecalis pyrimidine biosynthetic genes in this clone (designated pKV48) are part of a larger cluster resembling that seen in Bacillus spp. Transposon insertions were isolated at a number of sites throughout the cluster and resulted in loss of the ability to complement E. coli auxotrophs. The DNA sequences of the entire pyrD gene of E. faecalis and selected parts of the rest of the cluster were determined, and computer analyses found these to be similar to genes from Bacillus subtilis and Bacillus caldolyticus pyrimidine biosynthesis operons. Five of the transposon insertions were introduced back into the E. faecalis chromosome, and all except insertions in pyrD resulted in pyrimidine auxotrophy. The prototrophy of pyrD knockouts was observed for two different insertions and suggests that E. faecalis is similar to **Lactococcus** lactis, which has been shown to possess two pyrD genes. A similar analysis was performed with the purL gene from E. faecalis, contained in another cosmid clone, and purine auxotrophs were isolated. In addition, a pool of random transposon insertions in pKV48, isolated in E. coli, was introduced into the E. faecalis chromosome en masse, and an **auxotroph** was obtained. These results demonstrate a new methodology for constructing defined knockout mutations in E. faecalis.

L3 ANSWER 6 OF 11 MEDLINE DUPLICATE 2
ACCESSION NUMBER: 95319326 MEDLINE
DOCUMENT NUMBER: 95319326 PubMed ID: 7596286
TITLE: Isolation of **Lactococcus** lactis nonsense suppressors and construction of a food-grade cloning vector.
AUTHOR: Dickely F; Nilsson D; Hansen E B; Johansen E
CORPORATE SOURCE: Department of Genetics, Christian Hansen's Laboratorium Danmark A/S, Horsholm.
SOURCE: MOLECULAR MICROBIOLOGY, (1995 Mar) 15 (5) 839-47. Journal code: MOM; 8712028. ISSN: 0950-382X.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
OTHER SOURCE: GENBANK-L35276; GENBANK-L35277
ENTRY MONTH: 199507
ENTRY DATE: Entered STN: 19950817
Last Updated on STN: 20000303
Entered Medline: 19950731

AB Nonsense suppressor strains of **Lactococcus** lactis were isolated using plasmids containing nonsense mutations or as revertants of a nonsense auxotrophic mutant. The nonsense suppressor gene was cloned from two suppressor strains and the DNA sequence determined. One suppressor is an ochre suppressor with an altered tRNA(gln) and the other an amber suppressor with an altered tRNA(ser). The nonsense suppressors allowed isolation of nonsense mutants of a lytic bacteriophage and suppressible auxotrophic mutants of L. lactis MG1363. A food-grade cloning vector based totally on DNA from **Lactococcus** and a synthetic polylinker with 11 unique restriction sites was constructed using the ochre suppressor as a selectable marker. Selection, following electroporation of a suppressible purine **auxotroph**, can be done on purine-free medium. The pepN gene from L. lactis Wg2 was subcloned resulting in a food-grade plasmid giving a four- to fivefold increase in lysine aminopeptidase activity.

L3 ANSWER 7 OF 11 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1994:696816 CAPLUS
DOCUMENT NUMBER: 121:296816
TITLE: Selection and biochemical studies of pyrimidine-requiring mutants of Lactobacillus plantarum
AUTHOR(S): Masson, A.; Kammerer, B.; Hubert, J. -C.
CORPORATE SOURCE: Laboratoire de Microbiologie et de Genetique, l'Universite Louis-Pasteur, Strasbourg, Fr.
SOURCE: J. Appl. Bacteriol. (1994), 77(1), 88-95
CODEN: JABAA4; ISSN: 0021-8847
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Mutagenesis and mutant enrichment in L. plantarum, a **lactic acid** bacterium used in silage, sauerkraut, and sausage fabrication, were studied. In optimal conditions, auxotrophic mutants

were obtained that permitted investigation of the de novo pyrimidine biosynthesis. Uracil-requiring mutants were characterized for their enzymic defects, in aspartate transcarbamylase, dihydroorotase, dihydroorotate dehydrogenase, orotidine monophosphate pyrophosphorylase, or in orotidine monophosphate decarboxylase. The 5 enzymic activities are totally repressed by uracil in the growth medium.

L3 ANSWER 8 OF 11 MEDLINE DUPLICATE 3
ACCESSION NUMBER: 93322317 MEDLINE
DOCUMENT NUMBER: 93322317 PubMed ID: 7687248
TITLE: Gene inactivation in **Lactococcus** lactis: histidine biosynthesis.
AUTHOR: Delorme C; Godon J J; Ehrlich S D; Renault P
CORPORATE SOURCE: Laboratoire de Genetique Microbienne, Institut National de la Recherche Agronomique, Jouy en Josas, France.
SOURCE: JOURNAL OF BACTERIOLOGY, (1993 Jul) 175 (14) 4391-9. Journal code: HH3; 2985120R. ISSN: 0021-9193.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
OTHER SOURCE: GENBANK-M90760
ENTRY MONTH: 199308
ENTRY DATE: Entered STN: 19930826
Last Updated on STN: 19960129
Entered Medline: 19930813

AB **Lactococcus** lactis strains from dairy and nondairy sources were tested for the ability to grow in the absence of histidine. Among 60 dairy strains tested, 56 required histidine, whereas only 1 of 11 nondairy strains had this requirement. Moreover, 10 of the 56 auxotrophic strains were able to grow in the presence of histidinol (Hol+), the immediate histidine precursor. This indicates that adaptation to milk often results in histidine auxotrophy. The histidine operon was detected by Southern hybridization in eight dairy auxotrophic strains tested. A large part of the histidine operon (8 kb, containing seven histidine biosynthetic genes and three unrelated open reading frames [ORFs]) was cloned from an **auxotroph**, which had an inactive hisD gene, as judged by its inability to grow on histidinol. Complementation analysis of three genes, hisA, hisB, and hisG, in Escherichia coli showed that they also were inactive. Sequence analysis of the cloned histidine region, which revealed 98.6% overall homology with that of the previously analyzed prototrophic strain, showed the presence of frameshift mutations in three his genes, hisC, hisG, and hisH, and two genes unrelated to histidine biosynthesis, ORF3 and ORF6. In addition, several mutations were detected in the promoter region of the operon. Northern (RNA) hybridization analysis showed a much lower amount of the his transcript in the auxotrophic strain than in the prototrophic strain. The mutations detected account for the histidine auxotrophy of the analyzed strain. Certain other dairy auxotrophic strains carry a lower number of mutations, since they were able to revert either to a Hol+ phenotype or to histidine prototrophy.

L3 ANSWER 9 OF 11 MEDLINE DUPLICATE 4
ACCESSION NUMBER: 93322316 MEDLINE
DOCUMENT NUMBER: 93322316 PubMed ID: 8331070
TITLE: Gene inactivation in **Lactococcus** lactis: branched-chain amino acid biosynthesis.
AUTHOR: Godon J J; Delorme C; Bardowski J; Chopin M C; Ehrlich S D; Renault P
CORPORATE SOURCE: Laboratoire de Genetique Microbienne, Institut National de la Recherche Agronomique, Jouy en Josas, France.
SOURCE: JOURNAL OF BACTERIOLOGY, (1993 Jul) 175 (14) 4383-90. Journal code: HH3; 2985120R. ISSN: 0021-9193.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
OTHER SOURCE: GENBANK-M90761
ENTRY MONTH: 199308
ENTRY DATE: Entered STN: 19930826
Last Updated on STN: 19930826
Entered Medline: 19930813

AB The **Lactococcus** lactis subsp. lactis strains isolated from dairy products are auxotrophs for branched-chain amino acids (leucine, isoleucine, and valine), while most strains isolated from nondairy media are prototrophs. We have cloned and sequenced the leu genes from one **auxotroph**, IL1403. The sequence is 99% homologous to that of the prototroph NCDO2118, which was determined previously. Two nonsense mutations and two small deletions were found in the **auxotroph**

sequence, which might explain the branched-chain amino acid auxotrophy. Nevertheless, the leu genes from the **auxotroph** appear to be transcribed and regulated similarly to those from the prototroph.

L3 ANSWER 10 OF 11 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1964:40520 CAPLUS
DOCUMENT NUMBER: 60:40520
ORIGINAL REFERENCE NO.: 60:7178d-e
TITLE: Biosynthesis of diaminopimelic acid. XIII. Use of glycerol-1,3-14C, and **lactic acid** -114C- and -214C
AUTHOR(S): Angulo, J.; Madariaga, M. A.; Municio, A. M.
CORPORATE SOURCE: Univ. Madrid
SOURCE: Anales Real Soc. Espan. Fis. Quim. (Madrid) Ser. B (1963), 59(3), 221-30
DOCUMENT TYPE: Journal
LANGUAGE: Unavailable
AB The distribution of the radioactivity in the diaminopimelic acid synthesized in the presence of 14C-labeled glycerol and **lactic acid** by growing or resting cells of a lysine **auxotroph** of E. coli is consistent with the operation of the glyoxylic acid cycle and of a mechanism for the fixation of CO2. In addn., analysis of the distribution of the radioactivity in the proteins indicates the presence of distinct mechanisms for the biosynthesis of the diaminopimelic acid of the cell wall and of that of proteins.

L3 ANSWER 11 OF 11 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1961:119239 CAPLUS
DOCUMENT NUMBER: 55:119239
ORIGINAL REFERENCE NO.: 55:22488h-i
TITLE: L-Valine production using microbial **auxotroph**
AUTHOR(S): Nakayama, Kiyoshi; Kitada, Sohei; Kinoshita, Shukuro
CORPORATE SOURCE: Kyowa Hakko Kogyo Co., Ltd.
SOURCE: J. Gen. Appl. Microbiol. (Tokyo) (1961), 7, 52-69
DOCUMENT TYPE: Journal
LANGUAGE: Unavailable
AB Isoleucineless and leucineless mutants of glutamic acid-producing bacteria accumulated up to 8.75 mg. and 7.13 mg. L-valine/ml., resp., only when the corresponding amino acid had been added to the fermentation media at low concns. (30-250 .gamma. DL-isoleucine/ml. and 1-15 .gamma. L-leucine/ml., resp.). Higher concns. of these acids lowered the valine synthesis (by inhibiting the valine-synthesizing enzyme), but brought about an increase in **lactic acid** production esp. when biotin was also present.

09/715,479 Search Strategy/Results

YOU HAVE REQUESTED DATA FROM 6 ANSWERS - CONTINUE? Y/(N):y

L9 ANSWER 1 OF 6 MEDLINE DUPLICATE 1
ACCESSION NUMBER: 95319326 MEDLINE
DOCUMENT NUMBER: 95319326 PubMed ID: 7596286
TITLE: Isolation of Lactococcus lactis nonsense suppressors and construction of a food-grade cloning vector.
AUTHOR: Dickely F; Nilsson D; Hansen E B; Johansen E
CORPORATE SOURCE: Department of Genetics, Christian Hansen's Laboratorium Danmark A/S, Horsholm.
SOURCE: MOLECULAR MICROBIOLOGY, (1995 Mar) 15 (5) 839-47. Journal code: MOM; 8712028. ISSN: 0950-382X.
PUB. COUNTRY: ENGLAND: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
OTHER SOURCE: GENBANK-L35276; GENBANK-L35277
ENTRY MONTH: 199507
ENTRY DATE: Entered STN: 19950817
Last Updated on STN: 20000303
Entered Medline: 19950731

AB Nonsense suppressor strains of Lactococcus lactis were isolated using plasmids containing nonsense mutations or as revertants of a nonsense auxotrophic mutant. The nonsense suppressor gene was cloned from two suppressor strains and the DNA sequence determined. One suppressor is an ochre suppressor with an altered tRNA(gln) and the other an amber suppressor with an altered tRNA(ser). The nonsense suppressors allowed isolation of nonsense mutants of a lytic **bacteriophage** and suppressible auxotrophic mutants of L. lactis MG1363. A food-grade cloning vector based totally on DNA from Lactococcus and a synthetic polylinker with 11 unique restriction sites was constructed using the ochre suppressor as a selectable marker. Selection, following electroporation of a suppressible **purine auxotroph**, can be done on purine-free medium. The pepN gene from L. lactis Wg2 was subcloned resulting in a food-grade plasmid giving a four- to fivefold increase in lysine aminopeptidase activity.

L9 ANSWER 2 OF 6 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1988:543562 CAPLUS
DOCUMENT NUMBER: 109:143562
TITLE: Construction of .DELTA.aroA his .DELTA.**pur** strains of Salmonella typhi
AUTHOR(S): Edwards, Mary Frances; Stocker, Bruce A. D.
CORPORATE SOURCE: Sch. Med., Stanford Univ., Stanford, CA, 94305, USA
SOURCE: J. Bacteriol. (1988), 170(9), 3991-5
CODEN: JOBAAY; ISSN: 0021-9193
DOCUMENT TYPE: Journal
LANGUAGE: English

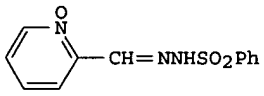
AB S. typhi Strains with 2 deletion mutations, each causing an attenuating **auxotroph**, have been constructed from strains Ty2 and CDC 10-80 for possible use as oral-route live vaccines. An aroA(serC)::Tn10 transposon insertion was first transduced from a Salmonella typhimurium donor into each wild-type S. typhi strain. Transductants of the Aro-SerC- phenotype were treated with transducing **phage** grown on an S. typhimurium strain with an extensive deletion at aroA; selection for SerC+ yielded transductants, some of which were .DELTA.aroA. A his mutation was next inserted into a .DELTA.aroA strain in each line by 2 steps of transduction. Two deletions affecting de novo purine biosynthesis were used as second attenuating mutations: .DELTA.purHD343, causing a requirement for hypoxanthine (or any other purine) and thiamine, and .DELTA.purA155, causing an adenine requirement. The purHD343 deletion was introduced into the .DELTA.aroA his derivs. of each strain by cotransduction with purH::Tn10, and the purA155 deletion was introduced into the CDC 10-80 .DELTA.aroA his deriv. by cotransduction with an adjacent silent Tn10 insertion by selection for tetracycline resistance. Tetracycline-sensitive mutants of each of the 3 .DELTA.aroA his .DELTA.**pur** strains were isolated by selection for resistance to fusaric acid. The tetracycline-sensitive deriv. of the CDC 10-80 .DELTA.aroA his .DELTA.purA155 strain, designated 541Ty, and its Vi-neg. mutant, 543Ty, constitute the candidate oral-route live-vaccine strains used in a recent volunteer trial (Levine, M. M., et al., 1987). Tetracycline-sensitive mutants of the .DELTA.aroA his .DELTA.purHD derivs. of strains Ty2 and CDC 10-80 may also be appropriate as live vaccines but have not been tested as such.

L9 ANSWER 3 OF 6 CAPLUS COPYRIGHT 2002 ACS DUPLICATE 2
ACCESSION NUMBER: 1985:484853 CAPLUS

DOCUMENT NUMBER: 103:84853
TITLE: Characterization of a Salmonella typhimurium mutant defective in phosphoribosylpyrophosphate synthetase
AUTHOR(S): Jochimsen, Bjarne U.; Hove-Jensen, Bjarne; Garber, Bruce B.; Gots, Joseph S.
CORPORATE SOURCE: Enzyme Div., Univ. Inst. Biol. Chem. B, Copenhagen, DK-1307, Den.
SOURCE: J. Gen. Microbiol. (1985), 131(2), 245-52
CODEN: JGMIAN; ISSN: 0022-1287
DOCUMENT TYPE: Journal
LANGUAGE: English

AB The isolation and characterization of a mutant (strain GP122) of S. typhimurium with a partial deficiency of phosphoribosylpyrophosphate (PRPP) synthetase activity is described. This strain was isolated in a purE deoD gpt **purine auxotroph** by a procedure designed to select guanosine-utilizing mutants. Strain GP122 had roughly 15% of the PRPP synthetase activity and 25% of the PRPP pool of its parent strain. The mutant exhibited many of the predicted consequences of a decreased PRPP pool and a defective PRPP synthetase enzyme, including: (1) poor growth on purine bases; (2) decreased accumulation of 5-aminoimidazole ribonucleotide (the substrate of the blocked purE reaction) under conditions of purine starvation; (3) excretion of anthranilic acid when grown in medium lacking tryptophan; (4) increased resistance to inhibition by 5-fluorouracil; (5) derepressed levels of aspartate transcarbamylase and orotate phosphoribosyltransferase, enzymes involved in the pyrimidine de novo biosynthetic pathway; (6) growth stimulation by PRPP-sparing compds. (e.g., guanosine, histidine); (7) poor growth in low-phosphate medium; and (8) increased heat lability of the defective enzyme. This mutant strain also had increased levels of GMP reductase. This genetic lesion, designated prs, was mapped by conjugation and **phage** P22-mediated transduction at 35 units on the Salmonella linkage map.

L9 ANSWER 4 OF 6 CAPLUS COPYRIGHT 2002 ACS DUPLICATE 3
ACCESSION NUMBER: 1979:533839 CAPLUS
DOCUMENT NUMBER: 91:133839
TITLE: Biochemical action of the antineoplastic agent 2-formylpyridine N-oxide benzenesulfonylhydrazone
AUTHOR(S): Lee, Sang He; Sartorelli, Alan C.
CORPORATE SOURCE: Sch. Med., Yale Univ., New Haven, CT, 06510, USA
SOURCE: Cancer Res. (1979), 39(7, Pt. 1), 2625-9
CODEN: CNREA8; ISSN: 0008-5472
DOCUMENT TYPE: Journal
LANGUAGE: English
GI



I

AB 2-Formylpyridine N-oxide benzenesulfonylhydrazone (I) [58809-82-8] caused in vitro prodn. of single-strand breaks in DNA of sarcoma 180 cells and inhibition of cell growth. The 50% growth-inhibitory concn. of freshly prepd. I for sarcoma 180 cells in culture after 24 h of exposure was 100 .mu.M. This level of drug caused damage to DNA, detected by alk. sucrose gradient centrifugation, within 15 min of treatment. I decompd. rapidly in aq. soln. with progressive loss of growth-inhibitory activity and ability to induce breakage of DNA. The majority of the damage to DNA induced by I was not repaired, even in 24 h after removal of I. In vitro studies with purified DNA from PM2 **phage** grown in Pseudomonas **thymidine auxotroph** BAL-31 imply that I induces damage to DNA which renders it labile to alk. hydrolysis. The damage to DNA produced by I correlates with cytotoxicity, suggesting that the lesions in DNA produced by I are responsible for its anticancer activity.

L9 ANSWER 5 OF 6 CAPLUS COPYRIGHT 2002 ACS DUPLICATE 4
ACCESSION NUMBER: 1974:35005 CAPLUS
DOCUMENT NUMBER: 80:35005
TITLE: Biosynthesis of .alpha.-putrescinylthymine in bacteriophage .phi.W-14-infected Pseudomonas acidovorans
AUTHOR(S): Kelln, Rod A.; Warren, R. A. J.
CORPORATE SOURCE: Dep. Microbiol., Univ. British Columbia, Vancouver, B. C., Can.

SOURCE: J. Virol. (1973), 12(6), 1427-33
CODEN: JOVIAM
DOCUMENT TYPE: Journal
LANGUAGE: English
AB The .alpha.-putrescinylthymine (putThy) in **bacteriophage** .vphi.W-14 DNA is synthesized at the mononucleotide level. It is labeled by uracil or deoxyuridine but not by thymidine, and it appears in the acid-sol. pool of infected cells before the onset of **phage** DNA synthesis. The methylene group at the C-5 position of the pyrimidine moiety of putThy is derived in vivo from a C1 unit. Exts. of a **phage**-infected **thymidine auxotroph** of the host, Pseudomonas acidovorans, apparently contain a **phage** -specific thymidylate synthetase and a **phage**-specific activity which forms 5-hydroxymethyl-dUMP from N5,N10-methylenetetrahydrofolate and dUMP.

L9 ANSWER 6 OF 6 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1971:1397 CAPLUS
DOCUMENT NUMBER: 74:1397
TITLE: Production of deletions in the chromosome of Escherichia coli
AUTHOR(S): Spudich, James A.; Horn, Virginia; Yanofsky, Charles
CORPORATE SOURCE: Dep. Biol. Sci., Stanford Univ., Stanford, Calif., USA
SOURCE: J. Mol. Biol. (1970), 53(1), 49-67
CODEN: JMOBAK
DOCUMENT TYPE: Journal
LANGUAGE: English
AB In E. coli B grown at 37.degree., essentially all spontaneous deletions detected by simultaneous mutation to resistance to the **bacteriophage** T1 and to tryptophan auxotrophy (tonB trpdel mutations) remove the entire trp operon (B-type deletions). In contrast, tonB trpdel mutations in E. coli K12 have a more random assortment of end points terminating within any of the 5 structural genes (K-type deletions) or beyond them. Chromosomal hybrids, constructed by transduction of the trp region from E. coli B into E. coli K12 (KB hybrids), could be divided into 2 distinct classes on the basis of their deletion patterns; some produced K-type deletions with the same relative frequency as in strain K12, while the others all produced the B-type and, without exception, at a 10- to 20-fold higher relative frequency than that obsd. in strain B. Further anal. of KB hybrids showed that the chromosomal region from E. coli B approx. midway between the cysB and trp operons is the region responsible for the generation of the B-type deletions characteristically found in this strain. Evidence suggested that the end points of the B-type deletions are not at fixed points in the chromosome. TonB trp deletions occurred in strains K12(W3110), B, and in the KB hybrids at 30, 37, and 42.degree., but in all these strains the relative frequency of deletions was reduced at 42.degree. more than 7-fold; the most pronounced redn., about 40-fold, was obsd. for the B-type deletions occurring in the KB hybrids. Protein synthesis at 37.degree. is required for production of the B-type deletions at the high frequency. The redn. in the generation of deletions when the cells are grown at 42.degree. may be due to temp. sensitivity of a protein. DNA replication is probably not required for the event that culminates in a B-type deletion since, under certain conditions, deletion events occur in a **thymidine auxotroph** apparently in the absence of thymidine. The effects of thymidine starvation, however, are complicated and depend on whether a preceding period of amino acid starvation is allowed. The bacterial recA and recB functions are not required for the production of the B-type deletions.

STIC-ILL

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Please provide the following references:

1) Inhibitory effects of 5-azacytidine and 5-azauridine in Escherichia coli
Doskocil, Jiri; Sorm, Frantisek
Collect. Czech. Chem. Commun. (1970), 35(6), 1880-91

2) Mode of action of 5-aza-2'-deoxycytidine in Escherichia coli
Doskocil, Jiri; Sorm, Frantisek
Eur. J. Biochem. (1970), 13(1), 180-7

3) Incorporation and phosphorylation of 5-azacytidine by normal and T4-phage-infected cells of E. coli.
Doskocil J; Sorm F
EUROPEAN JOURNAL OF BIOCHEMISTRY, (1969 Mar) 8 (1) 75-80.

4) Effect of nucleosides on the phosphorolysis of thymidine by normal and phage-infected cells of Escherichia coli
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6) Components of the nucleoside-transporting system in Escherichia coli
Doskocil, J.
Biochim. Biophys. Acta (1972), 282(1), 393-400

7) Erwina carotovora bacterial culture phage lysis prevention - involves introduction of 6-aza-cytidine into culture fluid in specified concn..******
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Patent Number: SU1439121
Publication date: 1988-11-23
Inventor(s): KISHKO YAROSLAV G (SU); CHERNETSKIJ VLADIMIR P (SU); ALEKSEEVA INNA V (SU); SPIVAK NIKOLAJ YA (SU); PETUKHOVA NAINA P (SU); SOLONTAJ FEDOR F (SU); MITKO VALENTINA S (SU)
Applicant(s):: INST MIKROBIOLOGII VIRUSOLOGII (SU); INST MOLEKULYARNOJ BIOLOG GENE (SU)
Requested Patent: SU1439121
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Priority Number(s): SU19853880275 19850109
IPC Classification: C12N1/00**

Thank you very much,

Eur. J. Biochem. 13 (1970) 180–187

The Mode of Action of 5-Aza-2′-Deoxycytidine in *Escherichia coli*

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5-Azadeoxycytidine is a good donor of deoxyribosyl group in reactions catalyzed by pyrimidine deoxyriboside phosphorylase, promoting the deoxyriboside-dependent incorporation of thymine by wild-type cells of *E. coli* about twice as effectively as either deoxycytidine or deoxyuridine. Prior deamination to 5-azadeoxyuridine is necessary for the utilization of the deoxyribose moiety of 5-azadeoxycytidine, since the deoxyribosyl-donor activity is entirely lost in mutants deficient in cytidine deaminase. Upon infection of wild-type cells with phage T4 the deamination of 5-azacytidine and 5-azadeoxycytidine is strongly depressed; a proportional loss of the deoxyribosyl-donor ability of the latter is observed while the activity of deoxycytidine remains unaffected.

In wild-type cells of *E. coli* 5-azadeoxycytidine inhibits protein synthesis and the replication of phage f2 as effectively as 5-azacytidine, but has much less effect on the replication of phage T4. In deaminase-less mutants 5-azadeoxycytidine has practically no inhibitory effects.

It is concluded that 5-azadeoxycytidine enters the cells of *E. coli via* deamination, followed by phosphorolytic cleavage of the glycosidic bond. Direct utilization of the deoxynucleoside, conserving the glycosidic bond and the 6-aminogroup, does not occur to any appreciable extent. In accord with this finding the biological effects of 5-azadeoxycytidine are similar to those of 5-azauridine but different from the action of 5-azacytidine.

5-Aza-2′-deoxycytidine [1] is a potent bacteriostatic [2] and cancerostatic agent [3]. In mouse leukemic cells this compound competes with the incorporation of deoxycytidine and deoxyuridine into DNA [4]. In cultures of *E. coli*, however, preliminary experiments have indicated that 5-azadeoxycytidine interferes mostly with the messenger function of RNA, but has remarkably little effect on the synthesis of DNA of phage T4, while 5-azacytidine is a strong inhibitor of replication of the DNA of this phage [5]. The present experiments show that these effects of 5-azadeoxycytidine can be interpreted as being due to a peculiar mechanism of entry of the inhibitor into the bacterial cells.

METHODS

5-Azacytidine and 5-azadeoxycytidine were prepared in the Department of Chemical Syntheses of this Institute, following the methods previously described [1,6]. Cytidine was obtained from Hoffmann La Roche, Basle; deoxycytidine (A grade), thymidine (A grade) and deoxyuridine (B grade) were from Calbiochem.

[2-^{14}C]Cytidine (188 mC/mmole), [2-^{14}C]uridine (280 mC/mmole), [2-^{14}C]thymine (8.7 mC/mmole), [2-^{14}C]deoxycytidine (25 mC/mmole) and [^{14}C]leucine (uniformly labeled, 83 mC/mmole) were obtained from the Institute of Research and Application of Radioisotopes, Praha. All radioactive compounds were suitably diluted with non-radioactive materials to obtain a convenient specific radioactivity.

Two strains of *E. coli* B deficient in cytidine deaminase were kindly donated to us by Dr. Munch-Petersen, Copenhagen. One of them, 0018, required a pyrimidine for growth; the other, OK 408, had no requirements, but was also deficient in deoxyribomutase and purine nucleoside phosphorylase. An F$^+$ strain was derived from OK 408 by growing it together with F$^+$ strain 2027 (met$^-$, leu$^-$, lac$^-$) obtained from Dr. Werner Arber, Genève. Both strains of bacteria were grown on tryptone medium and the cultures mixed in a ratio donor/acceptor equal to 1/20. After 2 h of incubation at 37° with gentle shaking, the mixed culture was diluted 10^6 times and plated on a mineral agar with glucose. Single colonies were tested for their ability to give plaques with phage f2. All those isolated gave imperfect and very turbid plaques, but could be

Enzymes. Cytidine deaminase or cytidine aminohydrolase (EC 3.5.4.5); deoxycytidylate hydroxymethylase or 10-hydroxymethyltetrahydrofolate:deoxycytidine monophosphate hydroxymethyltransferase; dCTPase or dCTP nucleotidohydrolase (EC 3.6.1.12); endolysine or *N*-acetylmuramide glycanohydrolase (EC 3.2.1.17); β-galactosidase or β-D-galactoside galactohydrolase (EC 3.2.1.23); thymidine phosphorylase or thymidine:orthophosphate deoxyribosyltransferase (EC 2.4.2.4); deoxyribosyl transferase or nucleoside:pyrimidine (purine) deoxyribosyl transferase (EC 2.4.2.6); pyrimidine phosphoribosyl transferase (EC 2.4.2.9).

used for replication of phage f2 in a liquid medium. Lysis of the cells was completed by adding lysozyme (5 μg/ml) and EDTA (5 mM). The lysates were treated with chloroform. The deaminase-less character of the isolates was verified by growing them in a glucose-mineral salts medium in the presence of cytidine (50 μg/ml), measuring the absorbance of the culture fluid, clarified by filtration on nitrocellulose membrane filters and acidified with HCl (0.33 M). From the absorbance at 255 and 280 nm the extent of conversion of cytidine to uridine was calculated.

Most experiments with uninfected bacteria and with phage T4 were performed in liquid cultures in shaken flaks, using the medium of Spizizen as previously described [5]. For replication of phage f2 a Tris-medium with low content of phosphorus [7] was used in order to avoid complications connected with precipitation of calcium ions necessary for the absorption of phage. Potassium dihydrogen phosphate was added to obtain a concentration of 200 μM. Immediately before infection the same amount of calcium chloride was added. In all experiments with phages the multiplicity of infection was at least 5, but usually 10 viable phage particles per bacterial cell. The fraction of uninfected cells was negligible.

The incorporation of labeled bases, nucleosides or leucine into macromolecules was determined by precipitation with trichloroacetic acid and filtration on nitrocellulose membrane filters (Synpor, Synthesia, Uhříněves). The radioactivity was determined with a gas-flow counter Frieseke-Höpfner.

Deoxycytidylate hydroxymethylase was determined according to Dirksen *et al.* [8], deoxycytidine triphosphatase was determined spectrophotometrically according to Wiberg *et al.* [9], fractionating the reaction mixtures on columns of Dowex 1×2. Phage endolysine was determined as described by Salser, Gesteland and Bolle [10].

RESULTS

The Partition of Cytidine and Deoxycytidine in RNA and DNA

Our previous study concerning the deoxyriboside-dependent incorporation of thymine [11] by prototrophic strains of *E. coli* has shown that 5-azadeoxycytidine may serve as a deoxyribosyl donor similarly as natural deoxynucleosides [12]. This finding indicates that 5-azadeoxycytidine may enter the cells by a mechanism involving the cleavage of the glycosidic bond, liberating a free base and the deoxyribosyl radical, which may be either catabolized or utilized in transdeoxyribosylation reactions [13]. Lichtenstein, Barner and Cohen [14] have postulated a similar mechanism for the utilization of deoxycytidine, observing that added deoxycytidine equilibrated completely with labeled uracil in a pyrimidine-deficient strain of *E. coli*. In order to verify the validity of their conclusions for our wild-type strain not requiring pyrimidines, we performed similar experiments, using cytidine or deoxycytidine labeled in the base, in competition with an excess of unlabeled deoxycytidine and cytidine, respectively. The results of these experiments are given in Tables 1—3. Excess cold deoxycytidine depresses the total amount of cytidine incorporated by the cells but does not selectively affect the portion incorporated into DNA; the percent of cytidine label incorporated into DNA, which is low in uninfected host cells or early after infection with phage T4, but very high later after infection, remains remarkably insensitive to the presence of excess deoxycytidine (Table 1). Conversely, most of the label of deoxycytidine is incorporated into both RNA and DNA (Table 2). Excess cytidine depresses the incorporation into DNA as well as into RNA (Table 3); however, some degree of

Table 1. *Incorporation of cytidine into DNA and RNA and competition with deoxycytidine*
The bacteria were infected with phage T4 Simultaneously with the infection either [^{14}C]cytidine alone (1 μg/ml, 0.1 μC/ml) or labeled cytidine with unlabeled deoxycytidine (50 μg/ml) were added

Time	RNA		DNA		Proportion in DNA	
	Cyd	Cyd + dCyd	Cyd	Cyd + dCyd	Cyd	Cyd + dCyd
min	counts/min	counts/min	counts/min	counts/min	%	%
5	15980	6225	1340	645	7.7	9.4
10	19500	6710	8200	2280	29.6	25.4
15	22150	6170	19350	4910	46.6	44.3
20	20200	6820	27900	6140	58.0	49.0
25	19100	6526	32500	8975	62.8	57.8
30	17750	5100	35450	11950	66.6	70.0

Table 2. *Incorporation of deoxycytidine into RNA and DNA*
[^{14}C]Deoxycytidine (2.27 μg/ml, 0.005 μC/ml) was added and its incorporation into RNA and DNA was determined

Bacteria	Time	Incorporation into		Proportion in DNA
		RNA	DNA	
	min	counts/min	counts/min	%
Uninfected bacteria				
	5	1872	328	14.9
	10	3836	604	13.6
	15	6743	1037	13.3
	20	10142	1358	11.8
	25	14591	1929	11.6
Bacteria infected with phage T4				
	5	871	131	13.1
	10	1876	434	18.8
	15	2098	1612	43.5
	20	2050	2980	59.2
	25	1700	4230	71.2

Table 3. *Incorporation of deoxycytidine into DNA and RNA and competition with cytidine*
[^{14}C]Deoxycytidine (2 μg/ml) (0.0044 μC/ml), either alone or together with cytidine (50 μg/ml), was added to both uninfected and T4 phage-infected cultures of *E. coli*. The incorporation into RNA and DNA was determined

Bacteria	Time	RNA		DNA		Proportion in DNA	
		dCyd	dCyd + Cyd	dCyd	dCyd + Cyd	dCyd	dCyd + Cyd
	min	counts/min	counts/min	counts/min	counts/min	%	%
Uninfected bacteria	25	30837	2319	3620	1733	10.5	42.7
T4 phage-infected bacteria	25	3555	444	6292	1162	63.9	72.5

Table 4. *Incorporation of labeled cytidine, deoxycytidine and uridine into 5-hydroxymethylcytosine and thymine of T4 phage DNA*
The nucleosides labeled with ^{14}C were added to a culture infected with phage T4. Phage DNA was isolated, hydrolysed with concentrated formic acid at 170° for 1 h and the bases chromatographed in isopropanol-HCl. The zones of bases, visible under a Mineralite lamp, were cut out and their radioactivity determined in a liquid scintillation counter. Each analysis was run in duplicate

Nucleoside	Radioactivity of		Radioactivity in	
	5-hydroxymethylcytosine	thymine	5-hydroxymethylcytosine	Average
	counts/min		%	
[^{14}C]Cytidine	19704	32209	38.0	39.0
	26700	40108	40.0	
[^{14}C]Deoxycytidine	43792	74569	37.0	36.7
	72356	126746	36.3	
[^{14}C]Uridine	94330	147746	39.0	38.5
	90068	146938	38.0	

selectivity is observed, especially in uninfected cells, where the incorporation of deoxycytidine into DNA is less affected by cytidine than the incorporation into RNA. This result may indicate that a limited amount of deoxycytidine may enter the cells directly upon conservation of the glycosidic bond. This question was not followed further and the experiment represented in Table 3 is not sufficient to prove this possibility.

These experiments indicate that a complete equilibration between the ribo- and deoxyribonucleosides of cytosine is taking place; the ribo- and deoxyribonucleosides mutually compete in some stage preceding this equilibration.

In Table 4 the incorporation of cytidine, deoxycytidine and uridine into 5-hydroxymethylcytosine and thymine of T4-phage DNA is represented. From this experiment, again analogous to former finding of Lichtenstein *et al.* [14], we see that the same ratio of 6-oxo-to 6-aminopyrimidines of DNA is obtained irrespective of the form in which the labeled pyrimidine is administered; cytidine and deoxycytidine are partially deaminated and uridine, or its phosphorylated derivatives, partially aminated before entering DNA.

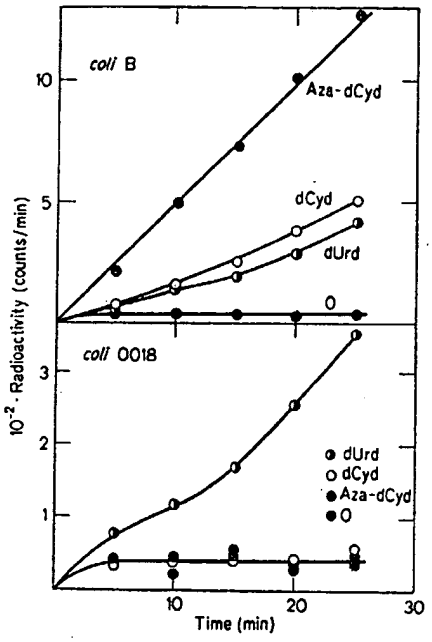

Fig. 1. *Incorporation of thymine by wild-type and deaminase-deficient strains of* E. coli *in the presence of different deoxynucleosides.* The concentration of [^{14}C]thymine was 3.3 μg/ml; that of deoxynucleosides, 50 μg/ml. Upper part, strain *E. coli* B; lower part, a deaminase-deficient strain 0018

The Inactivity of 5-Azadeoxycytidine in Strains Deficient in Cytidine Deaminase

To evaluate the significance of deamination for the uptake of 5-azadeoxycytidine the deaminase-less mutants of *E. coli* B were used, incapable of deaminating both cytidine and deoxycytidine. These experiments were prompted by the communication of Munch-Petersen [15], indicating that deoxycytidine cannot act as deoxyribosyl donor in such strains. The data of Fig. 1 confirm this finding and indicate that 5-azadeoxycytidine behaves in a similar manner, promoting the incorporation of thymine by a wild-type strain of *E. coli* about twice as much as cytidine or uridine, but being completely inactive in deaminase-less mutants. Deoxyuridine, however, is

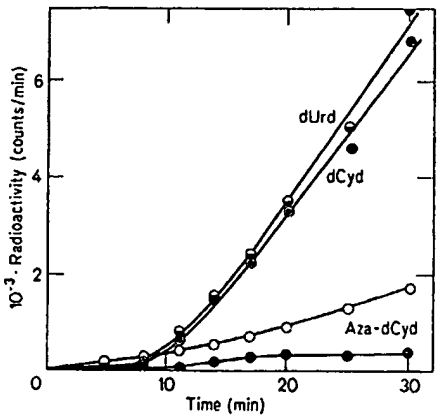

Fig. 2. *Incorporation of thymine in the presence of different deoxynucleosides by a culture of* E. coli *B infected with phage T4.* The conditions were the same as in Fig. 1

Table 5. *The deamination of cytosine and 5-azacytosine nucleosides by normal and T4 phage-infected cultures of* E. coli *B*

To a culture of *E. coli* B (5×10^8 cells/ml), growing on glucose-mineral salts medium, L-tryptophan (10 μg/ml) was added and one-half of the culture infected with 10 viable T4 phages per bacterial cell. Immediately after infection the nucleosides (50 μg/ml) were added to both aliquots. Aliquots of the cultures were acidified with HCl (final concentration 0.167 M) and filtered on membrane filters. The extent of deamination of cytidine and deoxycytidine was calculated from absorbancing at 280 and 250 nm, that of 5-azacytidine and 5-azadeoxycytidine from the readings at 255 and 240 nm

Nucleoside	Deamination after 30 min incubation	
	Normal culture	T4 phage-infected culture
	%	%
Cytidine	90	68
Deoxycytidine	84	66
5-Azacytidine	70	19
5-Azadeoxycytidine	74	16

equally effective in both the deaminase-less and wild-type strains.

If wild-type cells are infected with phage T4, both deoxycytidine and deoxyuridine still promote the incorporation of thymine, but 5-azadeoxycytidine is much less effective (Fig. 2). Table 5 shows that the deamination of 5-azacytidine and 5-azadeoxycytidine is strongly depressed upon infection with phage T4 while the deamination of cytidine and deoxycytidine is slightly affected. This observation provides another example of close connection between the rate of deamination and deoxyribosyl-donor ability of deoxycytidine and its analogs. Assuming that the deoxyribosyl-donor ability is proportional to the rate of uptake of the azapyrimidine moiety of the

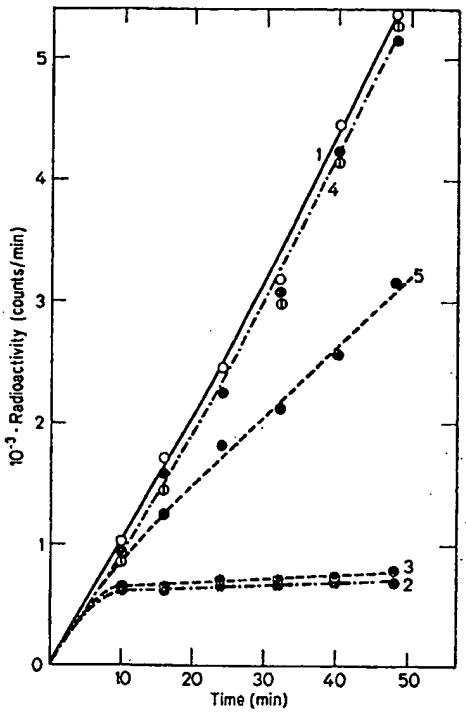

Fig. 3. *Incorporation of leucine by wild-type and deaminase-deficient strains of* E. coli *in the presence of 5-azacytidine and 5-azadeoxycytidine.* ○, control, *coli* B; ⊙, control, *coli* OK 408; ●, 5-azadeoxycytidine, 20 μg/ml; ●, 5-azacytidine, 20 μg/ml. Curves 1,2,3, wild-type strain *E. coli* B; curves 4 and 5, strain OK 408. The control of *E. coli* OK 408 has not been drawn since it was nearly identical with curve 1

compound, we may understand why 5-azadeoxycytidine is a rather inefficient inhibitor of replication of phage T4. However, the reasons why the infection with phage T 4selectively modifies the properties of host enzymes, remain obscure. Other examples of similar modifications of host enzymes by infection with T-even phages have been described [16, 17].

According to these findings 5-azadeoxycytidine enters the cell by deamination followed by trans-deoxyribosylation. We would therefore expect that the biological effects of this compound would resemble those of 5-azauridine. Furthermore, if this way of entry is exclusive and deamination is an obligatory step for the entry of the inhibitor into the cells, we could predict that the compound should be without any effect in a deaminase-less strain. The experiments to be described confirm both these assumptions.

In uninfected cultures of *E. coli* 5-azadeoxycytidine inhibits total protein synthesis as effectively as 5-azacytidine while in a deaminase-less strain the inhibition is hardly significant (Fig. 3). The synthesis of β-galactosidase in a glycerol medium is inhibited by 97% in *E. coli* B while 15% inhibition is observed

Table 6. *Inhibition of synthesis of β-galactosidase by 5-azadeoxycytidine and 5-azacytidine in* E. coli
The culture was induced with isopropylthiogalactoside (0.5 mM) and the enzyme assayed at 10 min intervals. The inhibitors (20 μg/ml) were added simultaneously with the inducer. The slopes of the production curves were expressed in per cent of the control

Medium	Inhibitor	Rate of synthesis of β-galactosidase	
		E. coli B	*E. coli* OK 408
		% of control	
Glycerol + casamino acids	5-Azadeoxycytidine	2.9	87.0
	5-Azacytidine	2.0	11.1
No carbon source	5-Azadeoxycytidine	72.5	69.0
	5-Azacytidine	15.3	69

completely removed by both cytidine and deoxycytidine, while only cytidine but not deoxycytidine is capable of completely removing the inhibitory effect of 5-azacytidine.

In a deaminase-less F+ strain 5-azacytidine still inhibits the replication of phage f2, although less effectively than in wild-type strains. 5-Azadeoxycytidine, however, has no inhibitory activity (Table 8).

The Effect of 5-Azadeoxycytidine on the Replication of Phage T4

In our earlier study 5-azacytidine has been shown to inhibit specifically the synthesis of T4 phage DNA, probably by interfering with the formation of 5-hydroxymethyl deoxycytidylate. No effect on the

Table 7. *Effect of inhibitors 5-azadeoxycytidine and 5-azacytidine on the production of phage f2 in the presence of cytidine and deoxycytidine*

Counteractors		Inhibitor	Effect of inhibitor			
			5-azadeoxycytidine		5-azacytidine	
Nucleoside	Concn.	Concn.	phage	production	phage	production
	μg/ml	μg/ml	counts	% control	counts	% control
None	0	none	8.8×10^{11}	100	6.4×10^{11}	100
None	0	10.0	1.7×10^{9}	0.19	7.2×10^{8}	0.11
Cytidine	2.5	10.0	8.1×10^{10}	9.16	2.4×10^{10}	3.75
	5.0	10.0	3.2×10^{11}	36.5	2.2×10^{11}	34.4
	10.0	10.0	4.3×10^{11}	49.4	4.7×10^{11}	73.7
	20.0	10.0	6.0×10^{11}	68.4	4.9×10^{11}	76.5
Deoxycytidine	2.5	10.0	4.1×10^{10}	4.6	1.8×10^{10}	2.8
	5.0	10.0	2.6×10^{11}	29.6	9.8×10^{10}	15.3
	10.0	10.0	5.9×10^{11}	67.0	1.4×10^{11}	21.8
	20.0	10.0	6.5×10^{11}	73.9	1.9×10^{11}	28.9

Table 8. *Effect of 5-azadeoxycytidine and 5-azacytidine on the production of phage f2 in a wild-type strain and deaminase-less strain*

Inhibitor	Production of phage f2			
	in strain 2027		in strain OK 408 F+	
	counts	%	counts	%
None	3.3×10^{11}	100	2.6×10^{11}	100
5-Azadeoxycytidine	3.3×10^{9}	0.11	2.2×10^{11}	86
5-Azacytidine	1.17×10^{9}	0.39	1.07×10^{10}	4.1

under the same conditions in the deaminase-less strain (Table 6).

5-Azadeoxycytidine inhibits the replication of phage f2 to the same extent as 5-azacytidine. The inhibitory effects of both compounds are different only in their sensitivity to the counteracting action of cytidine and deoxycytidine. Table 7 shows that the inhibition with 5-azadeoxycytidine is nearly

synthesis of early phage-specific proteins has been noted, but in the late period of phage replication the rate of protein synthesis has been found to drop to about 30% of the control. Proteins normally formed at this time, *e.g.* phage endolysine, are not formed in the presence of 5-azacytidine. As a result of these inhibitory effects the burst size of viable phage is reduced to about 0.1% of the control. Cytidine counteracts both inhibitory effects if added earlier than or simultaneously with 5-azacytidine; deoxycytidine, however, is incapable of restoring the normal burst size; in its presence, inhibition of DNA-synthesis persists while the rate of protein synthesis becomes normal (Fig. 4). Proteins formed under these conditions are predominantly of early character; this is evident from Table 9, showing a distinct overproduction of some early enzymes.

5-Azadeoxycytidine is a much weaker inhibitor of replication of phage T4, reducing the burst size to about 10—20% of the control. Synthesis of phage

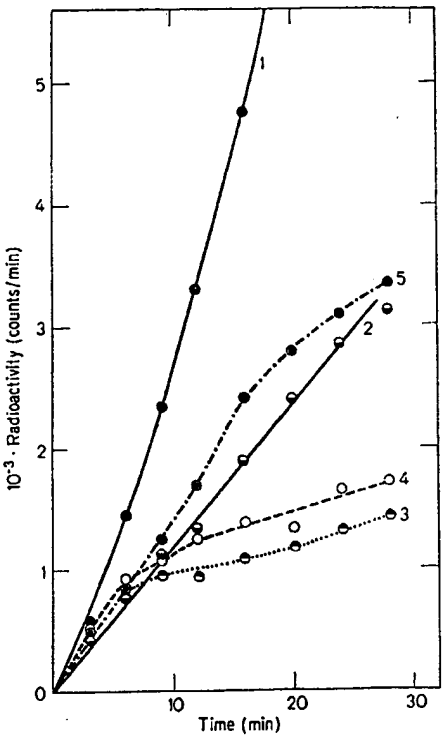

Fig. 4. *Incorporation of leucine by wild-type bacteria infected with phage T4.* 1, uninfected culture; 2, 3, 4, 5, cultures infected with phage T4; 2, no additions; 3, 5-azadeoxycytidine 4, 5-azacytidine; 5, 5-azacytidine and deoxycytidine. All inhibitors (20 μg/ml) were added simultaneously with the phage. The concentration of deoxycytidine (curve 5) was 50 μg/ml

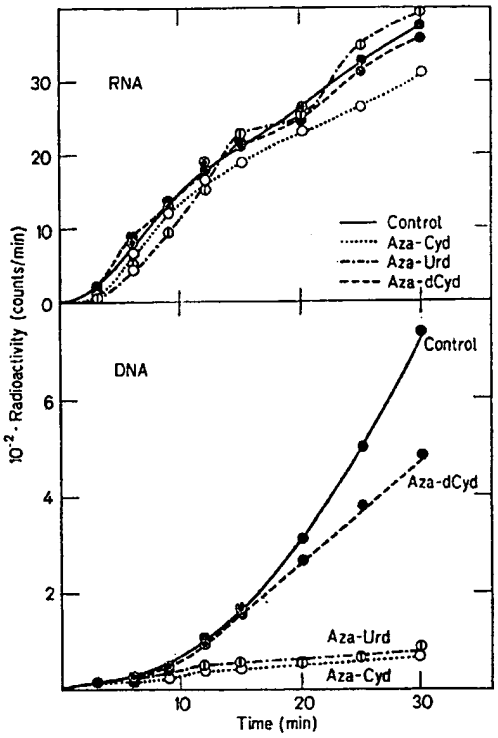

Fig. 5. *Effect of 5-azapyrimidine nucleosides on the synthesis of RNA and DNA in a culture of* E. coli *B infected with phage T4.* The incorporation of ^{32}P into alkali-sensitive and alkali-stable fractions was determined [5]. Upper part, RNA; lower part, DNA. The inhibitors (20 μg/ml) were added simultaneously with the infecting phage

Table 9. *Synthesis of some early enzymes of phage T4 in the presence of 5-azacytidine*
The concentration of 5-azacytidine was 10 μg/ml, that of deoxycytidine 50 μg/ml

Enzyme	Additions	Time	Activity
		min	% control
Deoxycytidylate hydroxymethylase	5-azacytidine and deoxycytidine	8	98
		14	132
		20	172
Thymidylate kinase	5-azacytidine	10	37
		25	98
	5-azacytidine and deoxycytidine	10	177
		25	234
Deoxycytidine triphosphatase	5-azacytidine	10	112
		25	200
	5-azacytidine and deoxycytidine	10	242
		25	254

Table 10. *Inhibition of synthesis of T4 phage endolysine by 5-azadeoxycytidine*
The concentration of the inhibitor was 50 μg/m

Additions	Time of addition	Phage titre[a]	Endolysine[b]	
			20 min	30 min
	min			
None	—	2.4×10^{11}	56.2	113
5-Azadeoxycytidine	0	3.8×10^{10}	0.8	7.1
5-Azadeoxycytidine	11	2.7×10^{10}	50.0	68.0

[a] Plated 1 hour after infection, completing the lysis with chloroform.
[b] Arbitrary units.

DNA is slightly affected (Fig. 5). However, protein synthesis in the late phase is inhibited in a similar manner as with 5-azacytidine (Fig. 4, Table 10). The rate of production of phage endolysine is reduced to about 10% of the control. Deoxycytidine completely counteracts all these inhibitory effects.

DISCUSSION

The experiments with strains deficient in cytidine deaminase indicate that deoxycytidine as well as 5-azadeoxycytidine enter the cells *via* deamination and uncoupling of the deoxyribose moiety. 5-Azauracil, transiently formed by deamination and

phosphorolytic cleavage of the glycosidic bond of 5-azadeoxycytidine, may enter the cell metabolism either after ribosylation with ribose-1-phosphate, followed by phosphorylation, or by a direct reaction with 5-phosphoribosyl-1-pyrophosphate catalysed by pyrimidine phosphoribosyltransferase. The latter reaction has been demonstrated to occur [18] in cell-free extracts of *E. coli*.

5-Azadeoxycytidine is taken up by the cells of *E. coli* by a mechanism analogous to the mode of entry of deoxycytidine and other analogs. Barth *et al.* [19] stated that deamination of deoxycytidine must precede its phosphorolysis, since deoxycytidine is not a substrate of pyrimidine deoxynucleoside phosphorylase. Tono and Cohen [20] have shown that arabinofuranosyluracil enters the cells of *E. coli* predominantly by phosphorolysis; direct phosphorylation without a cleavage of the glycosidic bond could be demonstrated to a very small extent with resting cells. In mammalian cells, however, direct phosphorylation of arabinosylcytosine has been found to predominate over deamination [21].

We may ask whether the outlined pathway is the exclusive way of entry of 5-azadeoxycytidine into the cells of *E. coli* or if some small portion may enter the cells with conservation of the glycosidic bond. The marginal inhibitory effects observed in deaminase-less strains cannot be taken as evidence in favor of this possibility. The 14% inhibition of replication of phage f2 in a deaminase-less strain is insignificant in comparison with the thousand-fold inhibition in wild-type strains. We may conclude that the cells of *E. coli* are incapable of taking up biologically significant amounts of 5-azadeoxycytidine by a mechanism not involving deamination.

The proposed pathway is also consistent with an earlier finding [22] that the action of 5-azadeoxycytidine may be counteracted by pyrimidine bases, while only nucleosides but not bases are effective counteractors of 5-azacytidine. This does not mean, however, that the action of 5-azadeoxycytidine is simply equivalent to that of 5-azauracil; we know, in fact, that the latter compound is much less active. This apparent discrepancy may probably be explained by a more efficient uptake of the deoxynucleoside in comparison with the base; evidence has been presented [23] indicating the existence of a transport mechanism, controlling the rate of uptake and transformation of a number of nucleosides and deoxynucleosides. Even if the deaminase and phosphorylase are located in the cell surface, they evidently are not accessible without the mediation of a transport mechanism. Therefore, 5-azauracil formed in the cells by deamination and phosphorolysis is much more effective than the same compound administered externally.

The peculiar biological effects of 5-azadeoxycytidine as well as the counteracting ability of deoxycytidine are in perfect agreement with the proposed pathway. The uncoupling of the deoxyribose moiety accounts for the lack of preferential interference with the metabolism of DNA. The obligatory deamination preceding phosphorolysis implies that the inhibitor enters the cells exclusively in the 6-oxo form unlike 5-azacytidine, which presumably may also be directly phosphorylated with preservation of the 6-amino group. In a later paper [24] evidence will be presented indicating that the strong inhibition of protein synthesis by 5-azacytidine is actually due to 5-azauridine produced by deamination while the inhibition of synthesis of phage DNA is a direct function of 5-azacytosine nucleotides. This finding provides a clue to the understanding of the effects of 5-azadeoxycytidine. Entering the cells exclusively in the form of derivatives of 5-azauracil, this compound strongly interferes with protein synthesis similarly as 5-azauridine, but is without appreciable effect on the synthesis of T4 phage DNA.

5-Azadeoxycytidine is a much weaker inhibitor of replication of phage T4 than 5-azacytidine. However, both compounds inhibit the synthesis of late phage proteins in a quite similar manner. In the case of 5-azacytidine this inhibition is preceded by nearly complete blocking of replication of phage DNA while with 5-azadeoxycytidine the synthesis of phage DNA is sligtly affected. Therefore, the lack of replication of DNA cannot be the cause of inhibition of late protein synthesis and the coincidence of the onset of inhibition of leucine incorporation with the derepression of late phage genes is probably accidental. It seems that the time of the rather sharp onset of inhibition of leucine incorporation is determined by the rate of uptake of the inhibitor by the cells. In uninfected cells mRNA synthesized in the presence of 5-azacytidine cannot be translated into functional protein; in a culture infected with phage T4, protein synthesis remains unaffected by 5-azacytidine for a period as long as 10 min, permitting normal synthesis of early phage enzymes. This difference probably is due to restricted uptake of the inhibitor by infected cells. The rate of phosphorylation and incorporation of 5-azacytidine is severely limited in comparison with uninfected cells [16] and the same is true with 5-azadeoxycytidine (*cf.* Fig. 1 and 2). Deoxyuridine, the uptake of which is probably unaffected by phage infection, was observed to cause a sharp inhibition of leucine incorporation beginning 4 min after phage infection [24].

The decrease of the rate of protein synthesis in cells infected with phage T4 thus seems to be a primary effect, independent of the preceding blockage of replication of phage DNA. On the other hand the quality of proteins synthesized undoubtedly is a function of previous replication of DNA. The overproduction of early enzymes and the lack of synthesis of phage endolysine, taking place in the presence of

5-azacytidine with excess deoxycytidine, seems to be a secondary phenomenon, being due to primary inhibition of DNA-replication. Preventing the replication of DNA by other means, *e.g.* by a genetic block or irradiation of phage with ultraviolet light [8,9], is known to have the same effect.

The ability of deoxycytidine to counteract some but not all inhibitory effects of 5-azacytidine also may be understood on the basis of the mode of entry of deoxycytidine into the cells, involving an obligatory deamination and uncoupling of deoxyribose. While capable of restoring the normal rate of protein synthesis, deoxycytidine cannot counteract the inhibitory effect of 5-azacytidine on the synthesis of DNA in T4-infected cells. This finding may be readily understood knowing that the inhibition of T4-DNA synthesis is due to 5-azacytosine phosphates formed by direct phosphorylation of 5-azacytidine, a pathway inaccessible to deoxycytidine. We may say that 5-azadeoxycytidine and deoxycytidine behave as a perfectly reciprocal pair of inhibitor-counteractor, interfering, negatively or positively, with protein synthesis, but incapable of interference with the synthesis of T4 phage DNA. This finding might seem logical if it were not for the fact that uridine derivatives entering the cells are partially aminated before being incorporated into T4 phage DNA (Table 4). We would therefore expect that even nucleosides entering the cell exclusively in the 6-oxo-form would, to a certain extent, acquire the inhibitory or counteracting properties of the 6-amino-derivatives. However, this type of inhibition or counteraction cannot be demonstrated. The reasons of this are not clear.

The present experiments show that the biological activity of inhibitors in different biological systems may be quite specific, being determined by the mechanism of their uptake by a particular type of cells. It seems that in mammalian cells, capable of direct phosphorylation of 5-azadeoxycytidine, this compound has quite different effects than in bacterial cells.

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Collect. Czech. Chem. Commun. (1970), 35(6), 1880-91

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Doskocil, Jiri; Sorm, Frantisek
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3) Incorporation and phosphorylation of 5-azacytidine by normal and T4-phage-infected cells of E. coli.
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7) Erwina carotovora bacterial culture phage lysis prevention - involves introduction of 6-aza-cytidine into culture fluid in specified concn..******
DERWENT CLASS: B04 D16
Patent Number: SU1439121
Publication date: 1988-11-23
Inventor(s): KISHKO YAROSLAV G (SU); CHERNETSKIJ VLADIMIR P (SU); ALEKSEEVA INNA V (SU); SPIVAK NIKOLAJ YA (SU); PETUKHOVA NAINA P (SU); SOLONTAJ FEDOR F (SU); MITKO VALENTINA S (SU)
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Requested Patent: SU1439121
Application Number: SU19853880275 19850109
Priority Number(s): SU19853880275 19850109
IPC Classification: C12N1/00**

Thank you very much,

THE INHIBITORY EFFECTS OF 5-AZACYTIDINE AND 5-AZAURIDINE IN *Escherichia coli*

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In cultures of wild-type strains of *E. coli* both 5-azacytidine and 5-azauridine completely inhibit the growth and total protein synthesis. In mutants deficient in cytidine deaminase only partial inhibition is observed with 5-azacytidine while 5-azauridine is as effective as in wild-type strains. However, the synthesis of β-galactosidase is inhibited or delayed by 5-azacytidine under conditions where little or no inhibition of total protein synthesis is observed. Both 5-azacytidine and 5-azauridine inhibit nearly completely the replication of T4 phage DNA if added simultaneously with the infecting phage; if added 8 min later 5-azacytidine affects the synthesis of phage DNA much more strongly than 5-azauridine. When 5-azauridine is added to a culture infected with phage T4 immediately after infection, the incorporation of leucine is arrested 4–5 min later. A similar treatment of a wild-type, T4-infected culture with 5-azacytidine produces a moderate decrease of leucine incorporation 9–14 min after the infection; in a deaminase-deficient, T4-infected culture 5-azacytidine has little effect on the incorporation of leucine, although the replication of phage DNA is inhibited as strongly as in wild-type T4-infected cells.

These findings confirm the notion that 5-azauridine formed by deamination of 5-azacytidine is responsible for the inhibition of total protein synthesis while the interference with the replication of T4 phage DNA is the primary function of 5-azacytidine itself. The relative insensitivity of protein synthesis in T4-infected cells to 5-azacytidine in comparison with protein synthesis in uninfected host is mainly due to a strong depression of deamination following the infection with T4 phage.

5-Azacytidine is a powerful inhibitor active in various living systems[1–5] and considerable effort has been spent to elucidate the mechanism of its diverse biological effects[6–8]. In cultures of *E. coli* 5-azacytidine is rapidly deaminated producing primarily 5-azauridine[9], and until recently it was not possible to resolve the direct effects of 5-azacytidine from those of 5-azauridine. The availability of mutants deficient in cytidine deaminase permits to investigate the monovalent effects of 5-azacytidine itself. The present communication is concerned with the action of 5-azacytidine and 5-azauridine on protein synthesis and replication of T4 phage DNA, comparing the response of wild-type and cytidine deaminase-deficient strains of *E. coli*.

EXPERIMENTAL

5-Azacytidine[10] and 5-azadeoxycytidine[11] were prepared in the Department of Organic Syntheses of this Institute. As a source of 5-azauridine a preparation of 5',6-anhydro-6-hydroxy 5,6-dihydro--5-azauridine[10] was used. When dispersed in ice-cold water or 2 mM acetic acid and diluted with 0·1M-HCl this preparation exhibited and absorption maximum at 241 nm ($\log \varepsilon = 3{\cdot}1$) indicating that about 15–20% of the compounds has been hydrolysed to produce 5-azauridine. Since all inhibitory effects of this preparation were completely counteracted by uridine, 5-azauridine is most probably the biologically active ingredient. All inhibitors were added to the cultures to produce a total concentration 20 μg/ml.

The deaminaseless strains, derived from *E. coli* B, were isolated by Dr O. Karström, Karolinska Institutet, Stockholm, and kindly donated to us by Dr A. Munch-Petersen, Copenhagen. In most experiments strains OK 408 was used. This strain had no growth requirements, but was also deficient in deoxyribomutase and purine nucleoside phosphorylase. It was repeatedly observed that glucose is not the best carbon source for this strain and some experiments were performed using a mineral salts medium[12] with glycerol instead of glucose. The bacteria were grown in shaken flasks at 37°C as previously described[4,5]. For experiments with phage T 4 the multiplicity of infection was 10 viable phage particles per bacterial cell. Tryptophan (25 μg/ml) was added immediately before infection.

The synthesis of β-galactosidase was induced by isopropylthio-β-D-galactoside (0·5 mM) in media with glycerol or lacking any carbon source. In the latter case, the bacteria were grown on glycerol medium, filtered on nitrocellulose membrane filters (50 ml culture, 2×10^8 cells/ml on Sartorius Membranfilter, 50 mm in diameter) and resuspended in the original volume of Spizizen medium without glucose. After 15 min shaking at 37°C, the inducer was added. The β-galactosidase was assayed using *o*-nitrophenyl-β-D-galactopyranoside (A grade, Calbiochem). The cells were lysed by shaking with toluene adding sodium deoxycholate (10 μg/ml) as described by Novick and Weiner[13].

Leucine-[^{14}C] (83 mCi/mmol) was added to the cultures together with non-radioactive L-leucine, obtaining final concentration 5 μg/ml and the radioactivity 0·2 μCi/ml. $KH_2^{32}PO_4$ (45 Ci/mg P) was obtained from Nuclear Centre, Amersham England.

RESULTS

Inhibition of Culture Growth

Fig. 1 shows that in the deaminase-deficient mutant the inhibition of growth by 5-azacytidine is only partial. The deaminase-deficient strain grows remarkably slowly on media with glucose (generation time 94 min as opposed to 38 min with the wild-type strain). Under these conditions the inhibitory effect of 5-azacytidine is hardly perceptible. In its presence the deaminase-deficient bacteria grow considerably more rapidly than the wild-type strain similarly treated, where the growth rate drops to 28% of the control 10 min after the addition and to 12% 50 min later. On glycerol medium the deaminase-deficient strain OK 408 grows nearly as rapidly as the wild-type strain (generation times 60 and 43 min, respectively). Here the growth rate of the wild-type strain is initially reduced to 8% of the control and decreases progressively; however, in a deaminase-deficient mutant growth proceeds in the presence

of 5-azacytidine at a rate equal to 34% of the control and this rate is constant for at least 2 hours showing no tendency to progressive decline.

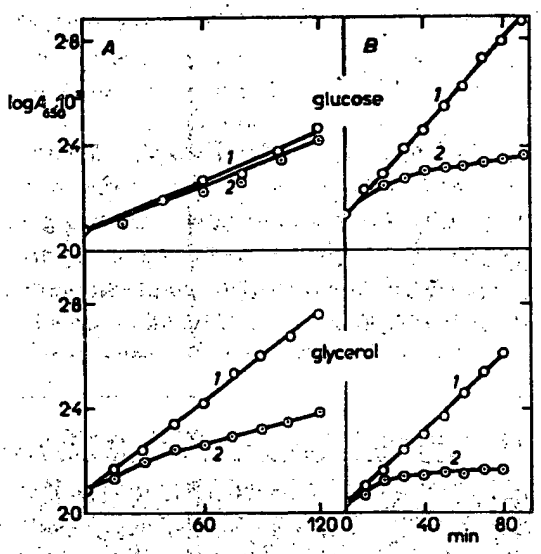


FIG. 1

Growth Curves of *E. coli* OK 408 (*A*) and *E. coli* B (*B*) on Spizizen Medium with Glucose and Glycerol

1 Control; *2* 5-azacytidine.

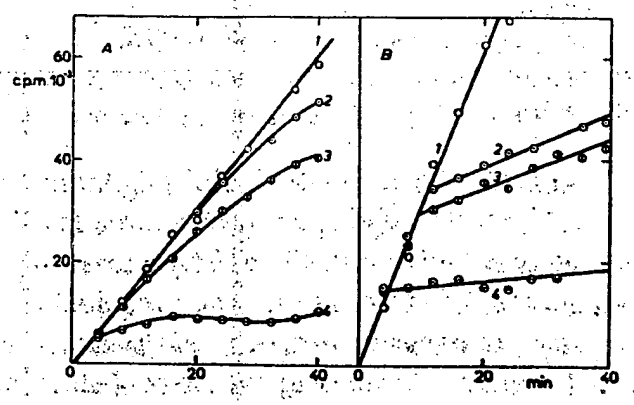


FIG. 2

Incorporation of Leucine-[^{14}C] by Cultures of *E. coli* in the Presence of 5-Azapyrimidine Nucleosides

Spizizen medium with glucose was used. *1* control; *2* 5-azadeoxycytidine; *3* 5-azacytidine; *4* 5-azauridine. *A E. coli* OK 408; *B E. coli* B.

Protein Synthesis

As previously reported[14] 5-azacytidine causes a mild inhibition of total protein synthesis in deaminaseless mutants contrasting with strong inhibition in wild-type strains. With 5-azauridine a strong inhibition is observed in deaminase-deficient as well as in wild-type strains. This finding is confirmed here (Fig. 2) and indicates that 5-azauridine is the active agent responsible for the sharp decline of protein synthesis. An aliquot treated with 5-azadeoxycytidine has been included in this experiment to show the basic similarity of its action with that of 5-azacytidine; deamination followed by uncoupling of the deoxyribose moiety has recently been shown[15] to be the only way of entry of this deoxynucleoside analog into the cells of *E. coli*. This

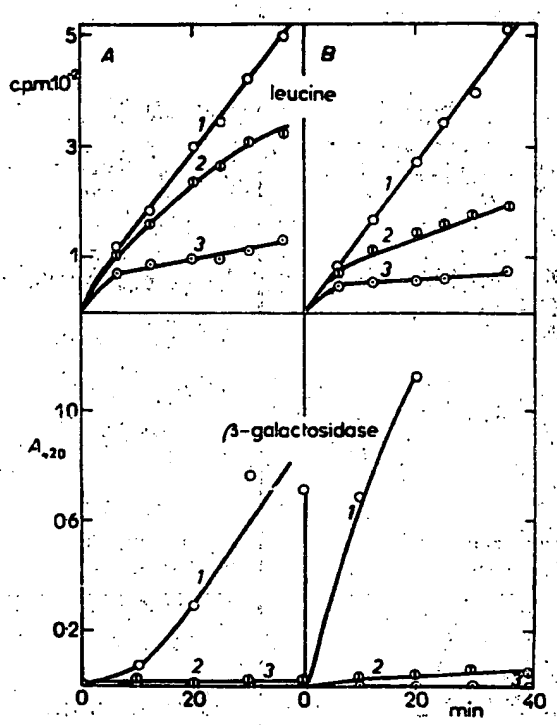


FIG. 3

Incorporation of Leucine and Synthesis of β-Galactosidase by *E. coli* Grown on Spizizen Medium with Glycerol

Leucine-[^{14}C], the inhibitors and isopropylthio-β-D-galactoside were added at zero time. *A E. coli* OK 408; *B E. coli* B; *1* control; *2* 5-azacytidine; *3* 5-azauridine.

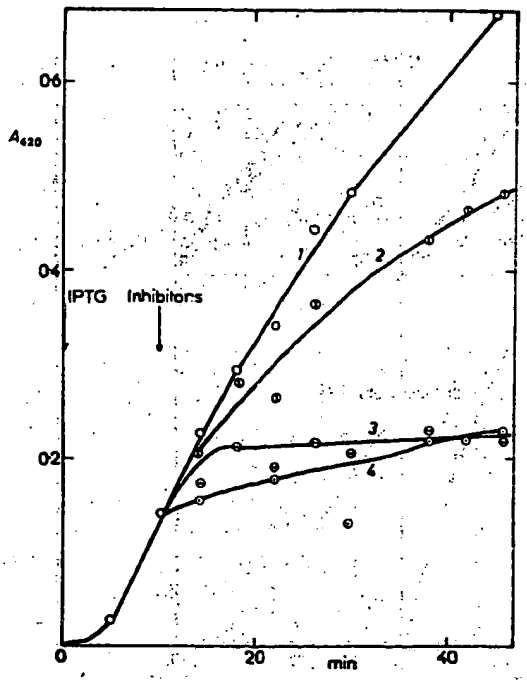


FIG. 4

Effect of 5-Azacytidine and 5-Azauridine on the Synthesis of β-Galactosidase by an Induced Culture of *E. coli* Deficient in Cytidine Deaminase

Isopropylthio-β-D-galactoside (ITG) was added at zero time, the inhibitors 10 min later. The bacteria, OK 408, were grown on Spizizen medium with glycerol and 0·25% casamino acids. *1* Control; *2* 5-azadeoxycytidine; *3* 5-azacytidine; *4* 5-azauridine.

is evident from the fact that in a deaminaseless strain 5-azadeoxycytidine is biologically inactive.

It will be noted that even in the wild-type strain the action of 5-azacytidine and 5-azadeoxycytidine is not exactly equivalent to the effect of 5-azauridine. With 5-azauridine the inhibition begins rather abruptly as early as 5 min after the addition while 10 min with 5-azacytidine and 12 min with 5-azadeoxycytidine have to pass before the inhibition of leucine incorporation becomes apparent. Also the slopes of the curves are different, indicating that the inhibition with 5-azauridine is more complete. The simplest explanation of this fact would be that some time is required until 5-azauridine accumulates in the medium in a concentration sufficient to bring about an inhibition. However, this interpretation is not sufficient, since 5-azacytidine probably is partially deaminated when entering the cells (see Discussion).

The experiment represented on Fig. 2 has been done using a glucose medium, where strain OK 408 grows very slowly. However, analogous experiments using glycerol media, where the growth rate is nearly normal, gave the same results (Fig. 3*A*).

In wild-type cells the arrest of total protein synthesis as measured by leucine incorporation is concurrent with the inhibition of synthesis of inducible enzymes such as β-galactosidase[4]. Knowing now that 5-azacytidine causes little inhibition of leucine incorporation in deaminase-deficient mutants, it was of interest to investigate the synthesis of β-galactosidase in these mutant strains. These experiments have shown that the parallelism between total protein synthesis and inducible enzyme synthesis is not conserved. Fig. 3 shows that the synthesis of β-galactosidase in glycerol medium is much more sensitive to the inhibitory action of 5-azacytidine than the incorporation of leucine. The same inhibition is observed when the inhibitors are added 10 min after the inducer, when the synthesis of β-galactosidase already has started (Fig. 4). Since the response of strain OK 408 to 5-azacytidine, *i.e.* inhibition of β-galactosidase synthesis without concurrent inhibition of total protein synthesis, seemed analogous to the effects of 5-fluorouracil, we suspected that catabolite repression[16] might be the cause of the inhibition observed. Therefore the synthesis of β-galactosidase was studied under conditions where catabolite repression should be excluded. Since strain OK 408 did not grow on succinate media described by Horowitz and Kohlmeier[16], the bacteria were grown on glycerol media and resuspended in fresh medium without any carbon source. An aliquot of the culture was treated with fluorouracil known to inhibit the synthesis of β-galactosidase through catabolite repression; therefore the lack of inhibition with fluorouracil should provide a good control proving that catabolite repression was reliably excluded. Fig. 5 shows that in wild-type strain 5-azacytidine still inhibits the synthesis of β-galactosidase while 5-azauridine is inactive; in deaminase-deficient mutant 5-azacytidine causes a temporary delay equal to about 15 min; after this period a normal rate of β-galactosidase synthesis is attained. It is remarkable that little or no inhibition with 5-azauridine is observed under these conditions; the interpretation of this result

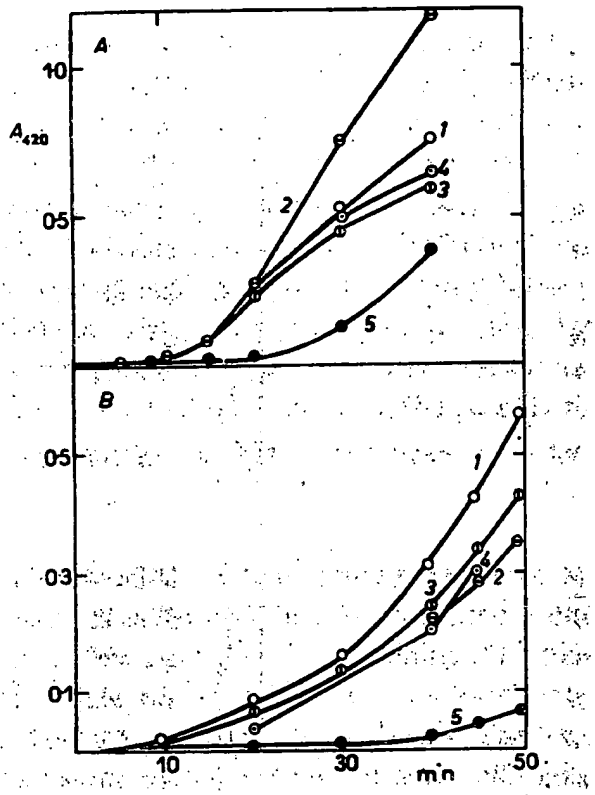


FIG. 5

Synthesis of β-Galactosidase in the Absence of Carbon Source

The bacteria were grown on glycerol medium, filtered and resuspended in Spizizen mineral salts medium without glucose. Isopropylthio-β-D-galactoside and the inhibitors were added at zero time. *A*, *E. coli* OK 408; *B*, *E. coli* B. *1* Control; *2* 5-fluorouracil (10 μg/ml) + thymidine 20 μg/ml; *3* 5-azadeoxycytidine; *4* 5-azauridine; *5* 5-azacytidine.

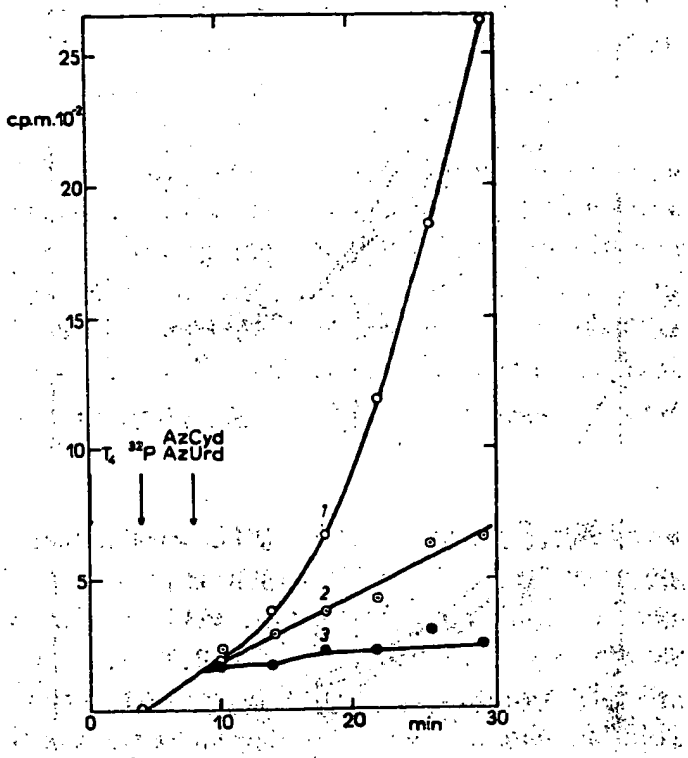


FIG. 6

Synthesis of DNA by a Culture of *E. coli* B Infected with Phage T4 in the Presence of 5-Azacytidine and 5-Azauridine

The bacteria were grown on a low-P medium, containing 6 μg/ml P. The culture was infected with phage T4. 4 min after infection $KH_2^{32}PO_4$ (1·4 μCi/ml) was added. The inhibitors (AzCyd, AzUrd) were added 8 min after infection. The incorporation of ^{32}P into alkali-stable fraction was measured. *1* Control; *2* 5-azauridine; *3* 5-azacytidine. The final phage titres, obtained 50 min after infection completing the lysis with chloroform, were the following:

Additions	Phage titre	% control
none	$1{\cdot}56 \times 10^{10}$	100
5-Azacytidine	$8{\cdot}8 \times 10^{8}$	5·6
5-Azauridine	$5{\cdot}8 \times 10^{8}$	3·7

is difficult at present. Certainly these experiments should be accompanied by measuring the rate of uptake and incorporation of 5-azacytidine and 5-azauridine by starving cells; it is possible that under conditions of absolute starvation 5-azauridine is not taken up by the cells while 5-azacytidine still is incorporated. Unfortunately, due to the complications connected with chemical synthesis of 5-azauridine, the labeled preparation was not available.

The Effect of 5-Azacytidine and 5-Azauridine on the Replication of Phage T4

As reported earlier[5], cells infected with phage T4 respond to 5-azacytidine in a manner entirely different from the response of uninfected cells: synthesis of phage DNA is specifically inhibited while the synthesis of phage-induced proteins is remarkably insensitive, at least from the quantitative point of view. The discovery of the divalent effects of 5-azacytidine has contributed to our understanding of these facts. The inhibition of replication of T4 phage has been shown to be as effective[14] in wild-type as in deaminase-deficient strains. Since the blockage of the replication of phage DNA is primarily responsible for the lack of phage production, we may

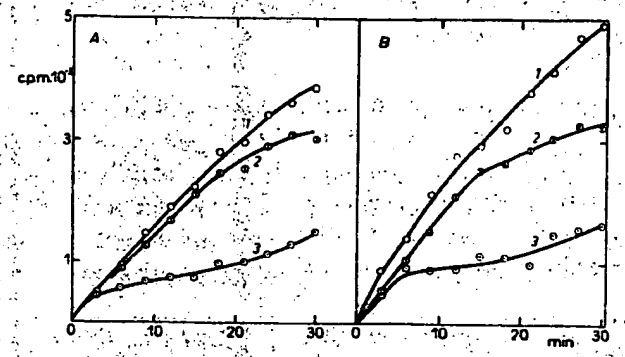


FIG. 7

Incorporation of Leucine-[^{14}C] by Cultures of *E. coli* Infected with Phage T4

The inhibitors and leucine-[^{14}C] were added to the cultures at zero time. *A*, *E. coli* OK 408; *B*, *E. coli* B. *1* Control, *2* 5-azacytidine; *3* 5-azauridine. The phage titres, obtained 75 min after infection completing the lysis with chloroform, were the following:

Additions	Phage titre		% control	
	OK 408	B	OK 408	B
None	$1{\cdot}32 \times 10^{10}$	$6{\cdot}05 \times 10^{10}$	100	100
5-Azacytidine	$5{\cdot}15 \times 10^{7}$	$5{\cdot}6 \times 10^{7}$	0·39	0·092
5-Azauridine	$3{\cdot}56 \times 10^{9}$	$3{\cdot}1 \times 10^{9}$	27·0	5·1

conclude that 5-azacytidine itself is capable of blocking the replication of phage DNA without requiring prior deamination. The experiments presented here were performed in order to show whether this effect is specific for 5-azacytidine or if 5-azauridine may produce a similar effect. Both 5-azacytidine and 5-azauridine block the synthesis of phage DNA if added simultaneously with the infection, while deoxy-5-azacytidine is slightly active[15]. However, if the analogs are added 8 min after infection, 5-azacytidine markedly decreases the rate of the synthesis of DNA while with 5-azauridine the synthesis of DNA continues at constant rate equal to that before the addition; however, the steep increase of the rate of DNA-synthesis, observed in the control, is prevented by 5-azauridine (Fig. 6).

Besides inhibiting the synthesis of phage DNA 5-azacytidine partially decreases the rate of protein synthesis beginning about 10 min after the infection. This effect has been reinvestigated using a deaminase-deficient strain. Fig. 7 shows that the course of protein synthesis in wild-type and deaminase-deficient, T4-infected cultures is not too different; 5-azauridine, however, brings about a strong inhibition of protein synthesis, beginning 3–5 min after infection. The remarkable similarity of the response of the deaminase-deficient and wild-type strains is understandable with respect to our recent finding[15], indicating that the rate of deamination of 5-azacytidine (as well as of 5-azadeoxycytidine) is strongly and selectively impaired by the infection with phage T4, while the rates of deamination of cytidine and deoxycytidine are slightly affected. Therefore, T4-infected cells resemble those organisms where the cytidine deaminase is not very active, so that 5-azacytidine itself is responsible for most inhibitory effects observed.

DISCUSSION

The present experiments show that deaminase-deficient bacteria may grow for a long time in the presence of 5-azacytidine, although at a reduced rate. These strains still incorporate labelled 5-azacytidine at a rate equal to about one-half the rate found in wild-type strains[14]. From previous studies[17] we know that 5-azacytidine is incorporated into all species of RNA as well as into DNA to an extent equivalent to about 30%–40% cytosine in newly formed RNA or DNA. In deaminase-deficient mutants the extent of replacement probably is not much less, since these mutants grow more slowly than wild-type strains, producing proportionally less nucleic acids per unit time. However, in spite of quite large extent of incorporation 5-azacytidine inhibits protein synthesis slightly. This implies that mRNA containing 5-azacytosine must be partly functional in protein synthesis. Furthermore we have to consider the fact that the growth, though slowed down to one-third of its normal rate, is still logarithmic for at least two hours in the presence of the inhibitor. This means that even stable forms of RNA, *i.e.* sRNA and rRNA, must be partly functional in spite of containing a large proportion of 5-azacytosine. In this respect 5-aza-

cytosine seems to be analogous to 5-fluorouracil, the incorporation of which into a ribopolynucleotide does not seriously impair its coding properties[18], although 5-fluorouracil has been found to be read as cytidine in an amber mutant of *E. coli*[19]. Similarly 5-azaguanine has been observed[20] to code correctly when substituting guanine in coding triplets. On the other hand, the presence of 5-azauracil in mRNA seems to make it nonfunctional in protein synthesis.

When attempting to explain this peculiar property of 5-azauridine its chemical instability may be considered first. 5-Azauridine indeed is much less stable than 5-azacytidine[21]. The first observable change is the hydration of the double-bond 5, 6, making the UV-absorbancy to disappear after about 1 h at 37°C, while a considerable portion of unchanged 5-azacytitine would persist as long as three days under the same conditions[7]. However, chemical change would account for the kinetics of inhibition observed only in case of 5-azauridine decomposing immediately after being incorporated into mRNA, *i.e.* at a speed much higher than the decomposition of free nucleoside or nucleoside phosphates in the intracellular pool. All curves of leucine incorporation show a very clear-cut, abrupt inhibition as early as 3–5 min after the addition of 5-azauridine, whereas a progressive increase of the inhibition would be expected if the rate of decomposition of the incorporated analog were of the same order of magnitude as that of free nucleoside. Low electron densities on the carbon atom 6 are responsible for the chemical instability of 5-azapyrimidines[7], making them susceptible to attacks by nucleophilic reagents. While static indices of electron densities are not too different in free bases, they may be modified if the bases become part of a polynucleotide structure. When engaged in base-pairing, 5-azauridine acts as a hydrogen acceptor while 5-azacytidine is a donor. Upon hydrogen bond formation the electrophilic character of carbon 6 of 5-azauracil would increase, *i.e.* the azapyrimidine ring would be destabilized; the contrary would be true of 5-azacytosine. Since the synthesis of mRNA involves base-pairing with DNA, we would expect a large part of 5-azauridylic acid to be hydrated in the very instant of its incorporation into the polynucleotide chain. On the other hand, most of 5-azacytidylic acid would remain unchanged. This hypothesis accounts very well for the different efficiency of both analogs in protein synthesis.

Another clue to understanding the strong specific inhibitory effects of 5-azauridine may be derived from the rapid reversion of inhibition taking place immediately after interrupting the influx of fresh inhibitor into the cells[4]. If nonfunctional mRNA were bound to the ribosomes, we would expect a lag of several minutes before fresh correct mRNA molecules would displace the faulty molecules with 5-azauracil. No such lag is observed and the simplest explanation of it is that the mRNA has not been bound to the ribosomes at all. The substitution of 5-azauracil in place of uracil in the codon AUG really could seriously affect its initiation function and such mechanism probably would account for the observed kinetics of inhibition and reversion.

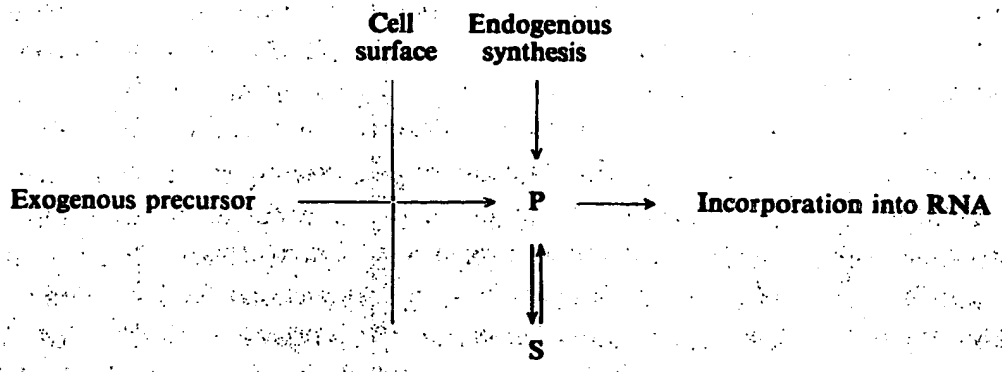
The inhibition of synthesis of β-galactosidase by 5-azacytidine in deaminaseless strains under conditions supposed to exclude catabolite repression[16] seems to indicate that 5-azacytidine may affect protein synthesis in some more subtle manner, without decreasing the overall rate of synthesis of polypeptides. Čihák, Veselá and Šorm[22] have observed that the frequency of larger polyribosomes is decreased by 5-azacytidine concurrently with inhibition of thymidine kinase in regenerating rat liver. Later Levitan and Webb[23] described a similar effect on polysomes, connected with the inhibition of synthesis of tyrosine transaminase in rat liver. These effects are probably due to 5-azacytidine itself, since its deamination by liver cells is slow. If the stability of polycistronic mRNA is affected by incorporation of 5-azacytidine or if the translation of such messenger molecules is prematurely terminated by an anomalous detachment of the translating ribosomes from the messenger tape, translation of genes located near the distal end of the operons would be partially inhibited. Such mechanism would bring about a disproportion of the amounts of enzymes of the same operon, without affecting too much the rate of total synthesis of proteins. To demonstrate the existence of such mechanisms the synthesis of several inducible enzymes belonging to the same operons should be studied.

The present investigations shed new light on the complex interaction of 5-azacytidine with the replication of phage T4. The inhibition of replication of phage DNA has been shown to be a primary function of 5-azacytidine. This action is irreversible[24]; short pulses of 5-azacytidine, given before or shortly after the infection, permanently reduce the capacity of the cells to replicate phage T4. The inhibition of synthesis of phage DNA by 5-azauridine is a consequence of the primary inhibition of synthesis of early phage-induced enzymes; this is apparent from Fig. 7, showing that the replication of DNA may proceed in the presence of 5-azauridine provided the early enzymes have already been formed. Though causing an early inhibition of synthesis of phage proteins, 5-azauridine is a much weaker inhibitor of the whole replication cycle. This is due to the fact that its action, unlike that of 5-azacytidine, is reversible, so that normal phage-protein synthesis is resumed as soon as 5-azauridine decomposes.

The remarkable insensitivity of T4 phage protein synthesis to 5-azacytidine, contrasting with strong inhibition in uninfected cultures, has been attributed to the fact that the rate of phosphorylation of 5-azacytidine is strongly reduced upon infection[24]. Recently we have found[15] that the deamination of 5-azacytidine, as well as of 5-azadeoxycytidine, is also strongly and selectively depressed by the infection with phage T4. Therefore, T4-infected cells are phenotypically similar to cells deficient in cytidine deaminase. We may now understand why the course of leucine uptake by wild-type and deaminase-deficient, T4-infected cultures is very similar, showing a weak and delayed inhibition. 5-Azadeoxycytidine, incapable of direct phosphorylation, cannot enter the cells unless deaminated[14] and this explains why this compound does not inhibit the replication of T4-phage DNA.

The irreversible nature of the inhibition of synthesis of T4 DNA, contrasting with the prompt reversibility of inhibition of protein synthesis, has been interpreted previously in terms of degradation of azacytidine-containing host DNA and mRNA, intoxicating the pool of nucleotides with 5-azacytidine phosphates. With the new knowledge available we will try to present a more detailed description of the fate of various forms of the inhibitor taken up by the cells. The model of RNA-precursor

pools according to McCarthy and Britten[25] has been found convenient for this purpose.



If a precursor of RNA is added to the medium, a large part of it will be incorporated into RNA with very little delay, passing through a very small pool, or a sequence of very rapid reactions, P. The rest of the precursor taken up by the cells enters a large pool, S, which slowly equilibrates with P. Pool S is responsible for slow incorporation of the labeled precursor into RNA after the precursor has been removed from the medium. The mean life time of the precursor in this pool (defined as a time required for the amount of labeled precursor in pool S to drop to 1/e its original value) varies according to the nature of the precursor; for cytosine it has been found equal to 21 min, for uridine, 9–12 min. A large part of cytosine passing through both pools, P and S, is incorporated into RNA as uracil – *i.e.* partial deamination is one of the very rapid reactions of the sequence P.

This model has been deduced from the experiments with bases, but its general features may apply for nucleosides as well. Let us assume that a short pulse of 5-azacytidine has been applied to a wild-type culture. From 5-azacytidine taken up by the cell in this interval, a part will be incorporated straight into RNA without any delay through pool P. Some portion of it will probably be subject to deamination when passing through the cell surface, so that both 5-azacytosine and 5-azauracil will be incorporated into RNA, even if there is no 5-azauridine in the medium. Another portion of total influx will flow over to pool S; here the ratio of 5-azauridine to 5-azacytidine will initially be the same as in the material incorporated through pool P. However, during the relatively long storage in pool S, 5-azauridine phosphates will decay rapidly while 5-azacytidine nucleotides will persist due to their greater stability. The deamination of 5-azacytosine nucleosides in pool S does not seem likely; the experiments of Buchwald and Britten[26] suggest that the deamination of cytosine derivatives occurs mainly on the cell surface whereas the opposite process, namely the amination of UTP to CTP, prevails in the interior of the cells. Consequently, the ratio 5-azauracil/5-azacytosine in the material incorporated from pool S will tend to decrease with time. Still another portion of the influx will undergo reduction to deoxynucleotides. From these, however, the deoxynucleotides of 5-azacytosine

but not those of 5-azauracil would find their way into DNA, since the 5-nitrogen atom of the latter obviously cannot be methylated by thymidylate synthase to yield an analog of thymidylic acid.

We see that 5-azauracil can be incorporated exclusively into RNA, mainly through the P pool, while the material incorporated through the S pool will predominantly contain 5-azacytosine. The nucleotides derived from breakdown of host DNA upon T4 phage infection will contain 5-azacytosine only. This situation agrees perfectly with the characteristics of inhibition of protein synthesis and T4 replication. 5-Azacytosine derivatives stored in the cells after a short pulse (namely in pool S and host DNA) are fully effective inhibitors of T4 replication while steady influx of the inhibitor from the medium is required for the inhibition of protein synthesis.

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Translated by the author (J. D.).

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1) Inhibitory effects of 5-azacytidine and 5-azauridine in Escherichia coli
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Collect. Czech. Chem. Commun. (1970), 35(6), 1880-91

2) Mode of action of 5-aza-2'-deoxycytidine in Escherichia coli
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Doskocil J; Sorm F
EUROPEAN JOURNAL OF BIOCHEMISTRY, (1969 Mar) 8 (1) 75-80.

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7) Erwina carotovora bacterial culture phage lysis prevention - involves introduction of 6-aza-cytidine into culture fluid in specified concn. ******
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Inventor(s): KISHKO YAROSLAV G (SU); CHERNETSKIJ VLADIMIR P (SU); ALEKSEEVA INNA V (SU); SPIVAK NIKOLAJ YA (SU); PETUKHOVA NAINA P (SU); SOLONTAJ FEDOR F (SU); MITKO VALENTINA S (SU)
Applicant(s):: INST MIKROBIOLOGII VIRUSOLOGII (SU); INST MOLEKULYARNOJ BIOLOG GENE (SU)
Requested Patent: SU1439121
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Priority Number(s): SU19853880275 19850109
IPC Classification: C12N1/00**

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The effect of nucleosides on the phosphorolysis of thymidine by normal and phage-infected cells of E. coli

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Received December 21, 1967

The importance of labeling of DNA with radioactive thymine and thymidine has inspired many investigations on this subject. (Munch-Petersen, 1967; Kammen and Strand, 1967). Thymidine usually is a much better precursor than thymine, but is often degraded to thymine by thymidine phosphorylase (Rachmeler et al., 1961). This is an inducible enzyme, but a considerable constitutive level is usually present in bacterial cells sufficient to cause a rapid cleavage of thymidine before the induced synthesis of the enzyme becomes apparent. For the same reason even the cells infected with phage T 4 degrade thymidine (Kammen and Strand, 1967), in spite of the absence of synthesis of bacterial inducible enzymes.

Boyce and Setlow (1962) observed that thymidine phosphorolysis in cultures of E. coli can be inhibited by deoxyadenosine. We have found that many other deoxynucleosides as well as ribonucleosides have the same effect. The phenomenon described here is distinct from that observed by Kammen and Strand (1967); in our experiments the added nucleosides or deoxynucleosides inhibit the phosphorolysis of thymidine and the high efficiency of incorporation is therefore due to a protection of thymidine rather than to the stimulation of thymine uptake.

Methods. The bacteria, E. coli B, were cultivated with shaking in a medium according to Spizizen (1958), containing 0.5% casamino acids. At a density 3 to 5 x 10^8 cells/ml the cultures were infected with a purified suspension of phage T4 (7.6 x 10^{11}/ml) in the presence of tryptophane (25 µg/ml), using 10 phage particles per bacterial cell. The incorporation of [^{3}H] thymidine (Radiochemical Centre, Amersham; specific activity 6 C/mmole) was followed by precipitating the samples with 5% trichloroacetic acid, filtering on nitrocellulose membrane filters and counting on a Frieseke-Hoepfner gas flow counter. In order to determine the extent of thymidine phosphorolysis the samples of the culture fluid were analysed by paper chromatography (Hotchkiss, 1948). The chromatograms were scanned using the gas flow counter fitted with an automatic recorder of radioactivity.

Results. In uninfected as well as in phage-infected cultures of E. coli the incorporation of thymidine (initial concentration 1-2 µg/ml) soon ceases as a result of its phosphorolysis to thymine. Addition of some ribo- or deoxyribonucleosides (usually 50 µg/ml) prevents the cessation of thymidine incorporation. Analyses of the medium, similar to that given in Tab. I and Fig. 1, indicate that the phosphorolysis of thymidine is inhibited by the added nucleosides. Pyrimidine nucleosides and deoxynucleosides as well as deoxyadenosine and adenosine are equally effective; deoxyguanosine is nearly inactive, increasing the level of the plateau slightly (Fig. 1). 6-Azacytidine and 6-azauridine are without effect, but 5-azacytidine is a strong inhibitor of phosphorolysis (Tab. II). In its presence uninfected cells incorporate thymidine normally (Doskočil, Pačes and Šorm, 1967), while in phage-infected cells

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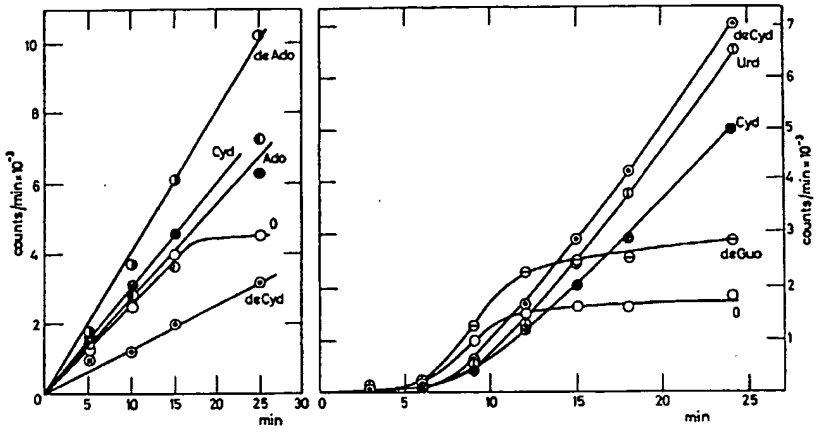

Fig. 1. Effect of Nucleosides on the Incorporation of ^3H Thymidine

Initial concentration of thymidine was 2 µg/ml. Nucleosides and deoxynucleosides were added at the concentration of 50 µg/ml. Left: uninfected cells; 25 min after addition of thymidine the media were analysed by chromatography and following values of conversion of thymidine were found: no addition, 100%; adenosine and cytidine, 0%; deoxyadenosine, 21%; and deoxycytidine, 15%. Right: cells infected with phage T4. Curves of incorporation of thymidine in the presence of deoxyuridine and deoxyadenosine (not shown) were similar to those of cytidine and uridine, while the addition of 6-azauridine and 6-azacytidine gave curves similar to that obtained without any added nucleoside. Phage titers obtained were 1.4 to 4.0 x 10^{10}/ml.

Table I

Conversion of Thymidine to Thymine by Infected and Uninfected Bacteria

One portion of the culture was infected with phage T4. 7 min after infection, both infected and uninfected portions (15 ml each) were filtered on a Millipore filter and resuspended in 3 ml of Spizizen medium without glucose. Thymidine (4 µC, 5 µg/ml) was added and the mixtures incubated, in separate 1-ml portions with added nucleosides, at 37°C without aeration for 5 min. The media were then analyzed by paper chromatography.

Additions	Percent conversion of thymidine	
	Infected bacteria	Uninfected bacteria
none	100	100
Cytidine, 200 µg/ml	14.2	20.8
Deoxycytidine, 200 µg/ml	11.8	17.7

incorporation of thymidine is strongly inhibited (Doskočil and Šorm, 1967).

In infected cells the added nucleosides and deoxynucleosides have small effects on the rate of incorporation of thymidine. This is evident from Fig. 1, when the slopes of the curves in the interval from 7 to 12 min, (i.e. before thymidine has

Table II

Inhibition of Phosphorolysis of Thymidine by 5-Azacytidine

[^{3}H]Thymidine (1 μC, 2 μg/ml) was added to 8 ml of a culture and 4 ml of it were infected with phage T4. Portions (2 ml) were taken from both infected and uninfected cultures and 5-azacytidine (50 μg/ml) was added. 15 min later the media were analyzed by paper chromatography.

Additions	Percent conversion of thymidine	
	Infected bacteria	Uninfected bacteria
none	100	90.1
5-Azacytidine, 50 μg/ml	18.5	22.9

decomposed) are compared. In uninfected cells, deoxyadenosine increases the rate of incorporation of thymidine by 50%, while deoxycytidine reduces this rate about two times (Fig. 1). The ribonucleosides prevent the phosphorolysis of thymidine, but are without effect on the rate of its incorporation.

The inhibition of phosphorolysis by nucleosides and deoxynucleosides is observed only with intact cells. In a cell-free system nucleosides have no appreciable effect (Tab. III). Similarly 5-azacytidine, a strong inhibitor *in vivo*, is inactive in a cell-free system (Doskočil, Pačes and Šorm,1967).

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Table III

Effect of Cytidine and Adenosine on the Phosphorolysis of Thymidine in a Cell-free Extract

To a bacterial culture (5×10^8 cells/ml) thymidine (50 µg/ml) was added as inducer of thymidine phosphorylase. 20 min later the bacteria were harvested by centrifugation, resuspended in 1 ml 0.1M phosphate, pH 6.0 and sonicated. The reaction mixture contained [^{3}H] thymidine, (5 µC, 1 µg), the nucleosides (25 µg) and the cell-free extract (0.02 ml) in 0.5 ml 0.1M phosphate, pH 6.0. Samples (0.1 ml) were taken in 10 min intervals and chromatographed.

Additions	Per cent conversion of thymidine	
	after 10 min	after 20 min
none	52.1	73.8
Cytidine, 50 µg/ml	48.4	62.2
Adenosine, 50 µg/ml	48.5	68.7

Discussion. The specificity pattern of the inhibitory action of nucleosides on thymidine phosphorolysis in vivo is entirely different from that of substrates and inducers of thymidine phosphorylase (Razzell and Casshyap, 1964). Deoxycytidine, deoxyuridine as well as the corresponding ribonucleosides are in vivo inhibitors, but only deoxyuridine is the substrate of the enzyme. Deoxyguanosine and deoxyadenosine are inducers, but only the latter has an inhibitory activity. The inhibition is limited to whole cells, infected or uninfected, while no comparable inhibition can be detected in cell-free extracts. Therefore direct interaction of the nucleosides with thymidine phosphorylase is probably not the cause of inhibition. The findings suggest the presence of a binding site on the surface of the cells, where thymidine has to be bound before the phosphorolysis can take place; this binding site could be susceptible to blocking by nucleosides. On the other hand the nucleosides or deoxynucleosides have surprisingly little effect on the rate of thymidine incorporation, especially

in phage-infected cells. Deoxycytidine, reducing the rate of incorporation of thymidine in uninfected bacteria, is without such effect in phage-infected cells, in spite of being a potential precursor of phage DNA.

These findings open a new way for improving the efficiency of labeling of DNA, namely by protecting radioactive thymidine against phosphorolytic breakdown by addition of a nucleoside.

A more detailed account concerning the action of pyrimidine nucleoside analogs will be published in Collection of Czechoslovak Chemical Communications.

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Please provide the following references:

1) Inhibitory effects of 5-azacytidine and 5-azauridine in Escherichia coli
Doskocil, Jiri; Sorm, Frantisek
Collect. Czech. Chem. Commun. (1970), 35(6), 1880-91

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Eur. J. Biochem. (1970), 13(1), 180-7

3) Incorporation and phosphorylation of 5-azacytidine by normal and T4-phage-infected cells of E. coli.
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EUROPEAN JOURNAL OF BIOCHEMISTRY, (1969 Mar) 8 (1) 75-80.

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5) The action of 5-azacytidine on bacteria infected with bacteriophage T4.
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6) Components of the nucleoside-transporting system in Escherichia coli
Doskocil, J.
Biochim. Biophys. Acta (1972), 282(1), 393-400

7) Erwina carotovora bacterial culture phage lysis prevention - involves introduction of 6-aza-cytidine into culture fluid in specified concn..******
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Publication date: 1988-11-23
Inventor(s): KISHKO YAROSLAV G (SU); CHERNETSKIJ VLADIMIR P (SU); ALEKSEEVA INNA V (SU); SPIVAK NIKOLAJ YA (SU); PETUKHOVA NAINA P (SU); SOLONTAJ FEDOR F (SU); MITKO VALENTINA S (SU)
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Application Number: SU19853880275 19850109
Priority Number(s): SU19853880275 19850109
IPC Classification: C12N1/00**

Thank you very much,

European J. Biochem. 8 (1969) 75—80

Incorporation and Phosphorylation of 5-Azacytidine by Normal and T4-Phage-Infected Cells of *E. coli*

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(Received October 10/November 16, 1968)

Upon infection with phage T4 the cells of *Escherichia coli* nearly completely lose their ability to phosphorylate and incorporate 5-azacytidine; at the same time, the phosphorylation of cytidine remains unaffected and its incorporation rate is decreased to about one-third. The same effect is observed if the infection takes place in the presence of chloramphenicol. Phage ghosts, even at a multiplicity of one particle per cell, selectively affect the phosphorylation of 5-azacytidine in a manner similar to the action of whole phage. Several peculiar features of the interference of 5-azacytidine with the replication of phage T4, contrasting with the effect on uninfected host cells, are made understandable by this finding.

Comparative studies have shown that in cultures infected with phage T4, 5-azacytidine acts in a manner completely different from the action on uninfected host [1,2]. In a healthy culture 5-azacytidine inhibits the synthesis of inducible enzymes when present in the period of mRNA synthesis [3]; in T4-infected cells, there is no inhibition of synthesis of early phage-specific proteins. Strong depression of production of viable phage is due to direct specific inhibition of synthesis of phage DNA; on the other hand the synthesis of DNA in uninfected cells is not affected. In order to elucidate the peculiar response of infected cells to 5-azacytidine, its phosphorylation and incorporation by normal and T4-phage infected cells was examined.

METHODS

5-[4-^{14}C]Azacytidine (4 mC/mmole) was prepared in this Institute by J. Morávek. [^{14}C]Cytidine (188 mC/mmole) was obtained from the Institute for Research and Application of Radioisotopes, Prague. [^{3}H]Thymidine (6 C/mmole) was a product of Radiochemical Centre (Amersham, England). Chloramphenicol was obtained from Spofa (Prague).

Phage T4 used for infecting the cultures was purified by differential centrifugation. Phage ghosts were prepared by osmotic shock according to Hershey and Chase [4], using a freshly purified suspension (titre before the shock 5.04×10^{12}/ml). Only 0.91 % plaque-forming particles remained after the treatment.

The bacteria, *E. coli* B, were grown on glucose-mineral salts medium according to Spizizen [5] supplemented with 0.25 % casamino acids. Tryptophan (50 μg/ml) was added to all cultures before infecting with phage T4 or with the ghosts; the control cultures, not intended for infection, received the same amount of tryptophan at the same time. The bacterial cultures (usually 3×10^{8} cells/ml) were infected with a purified suspension of phageT4, using 10 viable phages per bacterial cell. Three min after infection samples of the culture fluid were suitably diluted and plated for obtaining the counts of surviving bacteria. Less than 1 % bacteria remained uninfected. 45 min after infection the cultures were treated with chloroform and plated for phage counts.

The treatment with phage ghosts was performed in a similar manner, including the addition of tryptophan. The multiplicity of treatment was either (1—2) insufficient to kill most cells [6,7], or (7—10) reducing the colony-forming ability of the culture by 80—90 %.

The incorporation of 5-azacytidine and other radioactive compounds was determined by precipitating the samples with trichloroacetic acid and filtering on nitrocellulose membrane filters; the radioactivity was measured on a Frieseke-Hoepfner gas flow counter. The pools of phosphorylated intermediates (Fig. 2,4, Table 1) were determined in the following manner [8]. A bacterial culture containing tryptophan (50 μg/ml) was divided into aliquots (10 ml). One portion served as control while the others were infected with either the phage or phage ghosts at low or high multiplicity. Three min later samples were taken for plating the bacteria surviving the infection, and either 5-[^{14}C]azacytidine or [^{14}C]-cytidine (both at a concentration 4μg/ml) were added. After 3 min all cultures were rapidly filtered on nitrocellulose membrane filters (Sartorius, 50 mm in diameter) and the bacteria resuspended in 5 ml of cold

Unusual Abbreviations. mRNA, messenger RNA; tRNA, transfer RNA; rRNA, ribosomal RNA.

water. The filters were washed with additional 2 ml of water and discarded. The suspension of bacteria was immediately frozen. The bacteria were disrupted by sonication. The sonicate was again frozen and one-ninth volume of 50% trichloroacetic acid was added. After melting the suspension was filtered through a millipore filter, collecting the filtrate. Trichloroacetic acid was removed by extracting the filtrate 5 times with ether. The samples were then evaporated to a small volume and chromatographed in a mixture of *n*-butanol—acetic acid—water (10:1:3, v/v/v) on Whatman 1 paper. The dry chromatogram was scanned using a Frieseke-Hoepfner gas flow counter fitted with an automatic recorder of radioactivity. The phosphorylated derivatives of the radioactive nucleosides were detected as a radioactive zone on the starting point while the unchanged nucleosides moved apart. No attempt was made to separate the mono-, di- and triphosphates. The radioactive zones were cut out of the dry chromatograms and their radioactivity was determined using a liquid-scintillation counter Tri-Carb.

RESULTS

Rates of Incorporation of 5-Azacytidine by Healthy and Infected Cells

Fig. 1 shows that the rate of incorporation of 5-azacytidine is much lower in infected cultures than in uninfected portions of the same culture. If labeled azacytidine is added together with the infecting phage, the incorporation rate is as low as 3—5% of the value found in an uninfected control. If a culture is permitted to incorporate 5-azacytidine for several minutes and an aliquot of it is infected afterwards, the incorporation curve bends down about 1 min later and a small part of azacytidine incorporated before infection is progressively lost.

This effect may be specific for T4 phage, or possibly for the whole group of T-even phages. No decrease in the incorporation rate of 5-azacytidine was found when infecting a culture of *E. coli* C 600 with phage λ.

A decrease of the incorporation rate as a consequence of infection with T-even phages is a common phenomenon, observed with many natural precursors of nucleic acids [9, 10] and is caused by the cessation of synthesis of all species of nucleic acids of the host [11, 12]. Therefore it would not seem surprising to find 5-azacytidine behaving similarly. However, 5-azacytidine differs from normal precursors by the intensity of the effect. In comparable experiments, we found the incorporation rate of cytidine or uridine to drop to about 30% in consequence of phage infection (results not shown). Subsequent experiments demonstrate that the impairment of the incorporation of 5-azacytidine has a different cause, namely the alteration of the ability of the cells to phosphorylate this compound.

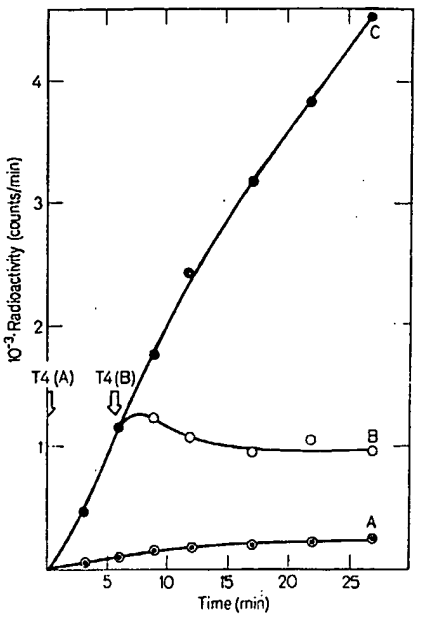

Fig. 1. *Incorporation of 5-azacytidine by uninfected and T4-phage infected cells.* At zero time, 5-[^{14}C]azacytidine (4 µg/ml) was added to a culture of *E. coli* B, and the culture was divided into three aliquots: (A) infected at zero time; (B) infected 6 min later; (C) uninfected. The final phage titers were 1.2×10^8/ml in A, 1.7×10^8/ml in B, and 4.9×10^{10}/ml in an untreated control

Table 1. *Incorporation of 5-azacytidine or cytidine by T4 phage-infected and uninfected cultures of* E. coli
The cultures were labelled with 5-[^{14}C]azacytidine (a, b) or cytidine (c, d) from 4 to 6 min after infection; a, c, cultures infected with 10 phage particles per cell; b, d, uninfected aliquots of the same culture. Chromatograms of trichloroacetic acid-soluble extracts are given on Fig. 2

Culture	Incorporation into the fraction precipitable with trichloroacetic acid		Incorporation into compounds soluble in trichloroacetic acid			
			Nucleosides		Nucleotides	
	counts/min	% control	counts/min	%	counts/min	%
a	81	3.5	1,865	45	1,421	7.7
b	2,321	100	4,115	100	18,564	100
c	47,321	51	5,134	81	205,324	119
d	92,740	100	6,330	100	172,331	100

Phosphorylation of 5-Azacytidine and Cytidine

Determining the pools of phosphorylated intermediates of 5-azacytidine, we found that in infected cells the pool size is negligible in comparison with healthy cells. In contrast, slightly higher pool sizes were found in infected cells using cytidine (Fig. 2). In this case the increase of the pool size in infected cells is understandable, considering the shut-off of syntheses of rRNA, tRNA, and host DNA (Table 1).

This experiment shows that the infection with phage T4 drastically alters the capability of the cells

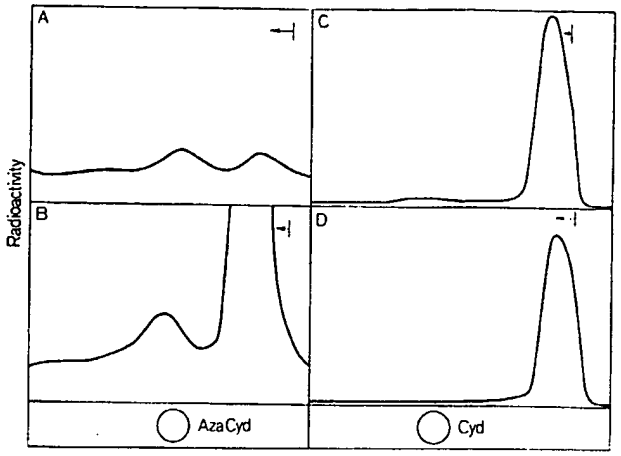

Fig. 2. *Chromatograms of trichloroacetic acid/soluble extracts from normal and infected cells.* Aliquots of an infected bacterial culture were treated with either 5-[^{14}C]azacytidine (A, B) or [^{14}C]cytidine (C, D) (both at concentrations 4 μg/ml) from the 4th to 6th min after infection; uninfected portions of the same culture were labeled in a similar manner. Extracts were prepared from all cultures by sonication and precipitation with trichloracetic acid as described in the text. A, B, 5-azacytidine; C, D, cytidine; A, C, culture infected with phage T4 at a multiplicity 10; B, D, uninfected controls. For other characteristics of the cultures see Table 1

to phosphorylate 5-azacytidine while the phosphorylation of cytidine is unaltered. It is noteworthy that the decrease of the pool of phosphorylated intermediates is not accompanied by a proportional increase of the pool of unchanged 5-azacytidine; we observe that this pool is approximately equal in infected as in uninfected cells. It seems that the diffusion transport of 5-azacytidine is low in comparison with active transport coupled with phosphorylation. However, it is also possible that the cells become impermeable to 5-azacytidine in consequence of phage infection. In any case the total amount of azacytidine taken up per unit time is much lower in infected cells.

The Effect of Chloramphenicol

The impairment of the ability of the cells to phosphorylate 5-azacytidine is concurrent with a number of other events brought about in host cells by phage infection [13]. Some of these effects are known to be caused merely by the absorption of the phage to host cells, while others require previous synthesis of phage-specific proteins. Chloramphenicol may be used to distinguish between these two groups of events [14—16]. Fig. 3 shows that the cessation of incorporation of 5-azacytidine by phage infection is not sensitive to chloramphenicol; we would conclude that protein synthesis is not required for the loss of the phosphorylation ability. However, the incorporation curves of the infected cultures with or without chloramphenicol are not exactly identical. Initially the incorporation of 5-azacytidine is slightly higher in chloramphenicol-treated cells; after about 4 min the incorporation curve bends down and the label

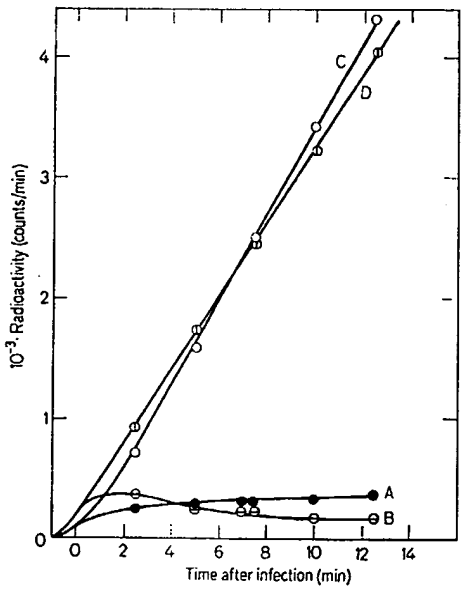

Fig. 3. *Effect of chloramphenicol on the incorporation of 5-azacytidine.* Chloramphenicol (100 μg/ml) was added to an aliquot of a bacterial culture. 6 min later 5-[^{14}C]azacytidine (4 μg/ml) was added to both the treated and untreated subcultures; one min later one-half of each subculture was infected with phage T4. A and B, infected; C and D, uninfected cultures; A and C, no chloramphenicol; B and D, with chloramphenicol

initially incorporated is progressively lost, while untreated infected cells continue to incorporate 5-azacytidine at a rate equal to about 3.3% of the uninfected control. This result suggests that a system responsible for incorporation in the host is completely

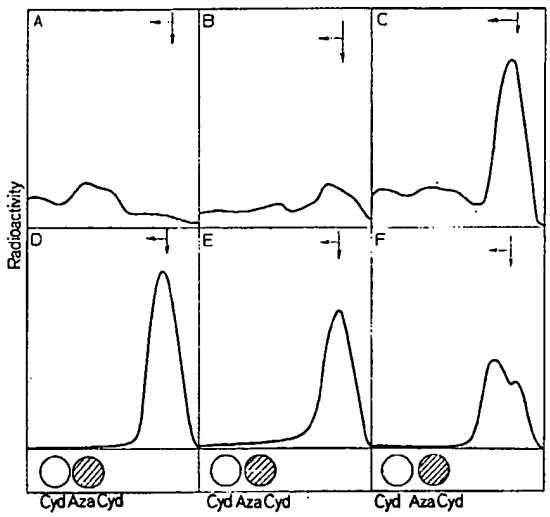

Fig. 4. *Chromatograms of trichloroacetic acid/soluble extracts from cultures treated with phage ghosts.* Portions of bacterial cultures were treated with T4 phage ghosts at different input ratios (see Table 2). Three min after the addition of ghosts either 5-[^{14}C]azacytidine or [^{14}C]cytidine (both at concentrations 4 μg/ml) were added; three min later all cultures were processed as described in the text; A—C, 5-azacytidine; D—F, cytidine. For other characteristics of the culture see Table 2

destroyed upon infection, while much less effective system is re-established during the first 4 min after infection by a process susceptible to chloramphenicol.

The Effect of Phage Ghosts

Finding the decline of the ability of phosphorylating 5-azacytidine not to be susceptible to chloramphenicol, we would expect it is caused merely by the absorption of phage to the cells. In this case phage protein should be capable of bringing about the same effect. The experiment represented on Fig. 4 confirms this assumption. The bacteria were treated with phage ghosts at two different input ratios; at low multiplicity (1—2 ghost particles per bacterial cell) the growth of the cells is only temporarily arrested (Table 2) and most cells recover while at high multiplicity most cells are killed [6, 7]. It is evident that even low input of phage ghosts, insufficient to kill the cells, is fully effective in destroying their capacity to phosphorylase 5-azacytidine; similarly as with whole phage, the phosphorylation of cytidine is not affected and higher pools are observed in cells treated with phage ghosts than in the control.

This experiment confirms that no synthesis of phage-specific proteins is required for the alteration of phosphorylation ability; absorption of phage protein is fully effective in this respect and one particle per cell is sufficient.

Table 2. *Characteristics of cultures treated with T4 phage ghosts*
The cultures were labelled with 5-[^{14}C]azacytidine (A—C) or with [^{14}C]cytidine (D—F) from 3 to 6 min after the addition of phage ghosts. Chromatograms of trichloracetic acid-soluble extracts are given on Fig. 4

Culture	Original counts	Input ratio of ghosts	Killing rate	Incorporation into the fraction precipitable with trichloroacetic acid		Incorporation into compounds soluble in trichloroacetic acid			
						Nucleosides		Nucleotides	
	cells/ml	ghosts/cell	%	counts/min	% control	counts/min	%	counts/min	%
A	7.08×10^8	7.13	82.3	87	1.7	3,244	45	502	1.3
B	7.08×10^8	1.41	20.0	1,312	25.7	829	11.4	9,317	24
C	7.08×10^8	none	none	5,109	100.0	7,275	100	38,589	100
D	4.3×10^8	11.7	84.0	33,434	30.7	10,797	95	204,996	170
E	4.3×10^8	2.34	44.0	89.212	82.7	3,850	34	153,149	127
F	4.3×10^8	none	none	107,919	100.0	11,335	100	121,363	100

Table 3. *Effect of pretreatment of bacteria with 5-azacytidine on the replication of phage T4*
At times indicated, samples were withdrawn from the culture for treatment with 5-azacytidine (20 μg/ml); the treatment was terminated by adding excess cytidine (200 μg/ml). Three min before infection samples were taken from all subcultures for plating; the bacterial counts were $3.1 \pm 0.3\times 10^8$ ml^{-1}. At zero time, all subcultures were infected

Time of addition of 5-azacytidine	Time of addition of cytidine	Phage yield	
min	min	ml^{-1}	%
No addition	— 0.5	6.6×10^{10}	100
— 1	— 0.9	3.9×10^{10}	59
— 1	— 0.5	1.3×10^{10}	19.7
— 5	— 1	2.5×10^9	3.7
— 10	— 6	3.2×10^9	4.9
— 20	— 16	3.6×10^9	5.5
— 30	— 26	3.9×10^9	5.6
+ 1	+ 5	4.9×10^9	7.4

Replication of Phage in Cultures Pretreated with 5-Azacytidine

In spite of very limited uptake by infected cells, 5-azacytidine is a potent inhibitor of replication of phage T4 [2]. When compared on the basis of the amount of 5-azacytidine taken up by the cells the replication of phage must be much more sensitive than any function of the host. From this point of view it seemed of interest to investigate the effect of short treatment with azacytidine, followed by phage infection. Table 3 gives an example of numerous experiments where 5-azacytidine was added at

various times before infection and its action interrupted either by adding excess cytidine or by filtering the culture on a nitrocellulose membrane filter and resuspending the cells in a fresh medium containing excess cytidine. These experiments have shown that a short treatment with azacytidine inhibits the replication of phage more effectively when given before infection than after it; surprisingly we see that the effectivity of treatment does not decrease significantly when the time interval between the treatment and infection is prolonged. Fig. 5 shows that a pre-treatment with 5-azacytidine inhibits

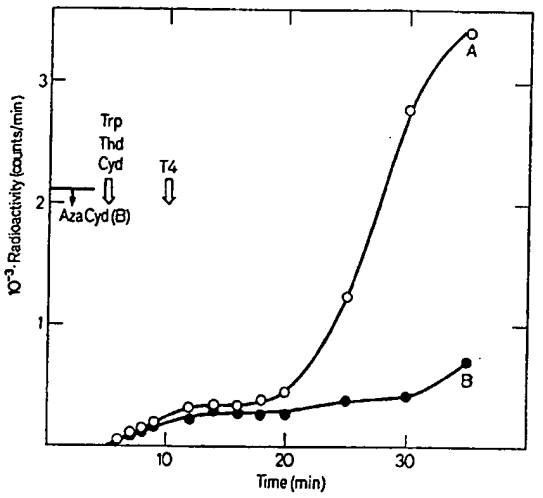

Fig. 5. *Effect of pre-treatment with 5-azacytidine on the incorporation of [^{3}H]thymidine before and after infection with phage T4.* One-half of a bacterial culture was treated with 5-azacytidine (20 μg/ml) for four min, rapidly filtered on a nitrocellulose membrane filter and resuspended in a fresh medium containing tryptophan (50 μg/ml), cytidine (50 μg/ml) and [^{3}H]thymidine (0.5 μC/ml, total concn. 2 μg/ml). The other half of the culture was processed in the same manner except that the treatment with 5-azacytidine was omitted. Five min later both cultures were infected with phage T4. A, untreated control, B, culture pre-treated with 5-azacytidine. The final yield of viable phage was 2.55×10^{10} in A and 4.8×10^9 in B

strongly the incorporation of thymidine after infection, in the period of synthesis of phage DNA, while the rate of incorporation of thymidine before infection is only slightly affected. It will be noted that cytidine (50 μg/ml) was present in both the treated culture and the untreated control: under these conditions the phosphorolysis of thymidine is inhibited [17,18] and the rate of its incorporation may be taken as truly indicating the synthesis of DNA. Under this assumption, the experiment indicates that the pre-treatment with azacytidine inhibits the subsequent synthesis of phage DNA.

DISCUSSION

The mechanism of inhibition of phage replication by 5-azacytidine is entirely different from its effect on host cells. In uninfected host 5-azacytidine inhibits protein synthesis, being incorporated into mRNA, while the rate of synthesis of DNA remains constant even for long periods of exposure [1]. In infected cells phage-DNA synthesis is strongly affected. The impairment of synthesis of phage DNA has been shown not to be a consequence of a primary inhibition of synthesis of early phage-specific enzymes. On the contrary, synthesis of these enzymes has been found rather insensitive to the action of azacytidine [2]. This latter finding is made quite understandable by the present results, showing the incorporation of azacytidine into RNA of infected cells to be negligible in comparison with whole cells.

In order to understand why the synthesis of phage DNA is inhibited by very low amounts of azacytidine taken up by the infected cells, we have to assume that much lower pools of phosphorylated derivatives of azacytidine are sufficient than those required for appreciable incorporation into mRNA and causing inhibition of protein synthesis. Alternatively, a possibility should be considered that the inhibition of phage-DNA synthesis does not require the phosphorylation of azacytidine, being due to the unchanged compound. Since we are not able to locate the site of the block responsible for the inhibition of phage-DNA synthesis, this question is difficult to answer.

Different sensitivity levels of the mechanisms responsible for inhibition of host protein synthesis and phage replication account for the latent irreversible effect of pre-treatment of the cells upon the production of phage. When exposed to a short pulse of azacytidine, healthy cells incorporate much more azacytidine than infected cells treated in a similar manner; yet the amount taken up by the latter is sufficient to inhibit phage replication. The remarkable persistence of the latent effect is concurrent with the retention of 5-azacytidine in uninfected cells. It has been shown [3] that 5-azacytidine, incorporated primarily into mRNA, cannot be removed from the cells by excess cytidine, being chased into rRNA, tRNA, and DNA. Even a limited turnover of these species may provide a persistent pool of azacytidine derivatives comparable to that formed upon treatment of infected cells and sufficient to inhibit the replication of phage. These considerations explain how a short treatment before infection can be more effective than a similar treatment applied to infected cells.

We may distinguish between the short-term reversible and long-term irreversible effects of 5-azacytidine. The former, being due to the incorporation of azacytidine into mRNA and manifesting itself by inhibition of protein synthesis and growth, is readily reversed as soon as the incorporation of

fresh azacytidine into mRNA is prevented; the irreversible effect, consisting of storage of azacytidine in rRNA, tRNA and DNA, is apparently harmless for host cells but becomes apparent on subsequent infection, causing a twenty-fold reduction of the capacity of replicating phage T4.

While accounting for some unusual features of the action of azacytidine on T4-infected bacteria, these findings pose a new problem—how the interaction of the cell surface with a single particle of phage protein can destroy selectively the ability to phosphorylate 5-azacytidine, leaving the phosphorylation of cytidine unaffected. It seems probable that the uptake and phosphorylation of 5-azacytidine is more complex than the phosphorylation of cytidine, requiring additional enzymes or a group of enzymes. If the cooperation of the necessary enzymes is disturbed by interaction of cell surface with phage protein, the observed selective impairment of azacytidine uptake may be understood.

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Publication date: 1988-11-23
Inventor(s): KISHKO YAROSLAV G (SU); CHERNETSKIJ VLADIMIR P (SU); ALEKSEEVA INNA V (SU); SPIVAK NIKOLAJ YA (SU); PETUKHOVA NAINA P (SU); SOLONTAJ FEDOR F (SU); MITKO VALENTINA S (SU)
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Requested Patent: SU1439121
Application Number: SU19853880275 19850109
Priority Number(s): SU19853880275 19850109
IPC Classification: C12N1/00**

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THE COMPONENTS OF THE NUCLEOSIDE-TRANSPORTING SYSTEM IN *ESCHERICHIA COLI*

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SUMMARY

A mutant of *Escherichia coli* resistant to 5-azacytidine was selected and shown to be deficient in the high-affinity component of the nucleoside-transporting system which participates in several types of conversion of nucleosides, such as incorporation and deamination of 5-azacytidine and cytidine as well as phosphorolysis of thymidine. All these reactions are affected in a similar manner when wild-type cells are infected with T4-phage or treated with osmotically disrupted phage, while the treatment of mutant bacteria with this phage does not significantly alter the value of the Michaelis constant for the phosphorolysis of thymidine. Since the metabolic conversion of nucleosides in mutant cells remains susceptible to competitive inhibition with heterologous nucleosides, it seems that another, low-affinity component participates in the transport, which is still active in mutant or phage-infected bacteria. Cytidine is transported predominantly by the low-affinity component, provided its concentration in the medium is sufficiently high; 5-azacytidine, however, requires the high-affinity component for efficient transport at any concentration.

INTRODUCTION

The transport of many nutrients and metabolites across the cell membrane is mediated by specific systems which may consist of several components with different functions and range of specificity[1-3]. Although the transport of pyrimidine bases and nucleosides has been studied extensively in yeast cells[4], little is known about nucleoside permeases in bacteria[5,6]. Selection for resistance to structural analogues of metabolites has often been used as a means of obtaining mutants deficient in specific permeases[7-10]. 5-Azacytidine, a structural analogue of cytidine, competitively inhibits the metabolic conversion of other nucleosides[11], presumably competing for a component of the nucleoside-permease system. We expected that deficiency in this component might render the cells resistant to 5-azacytidine. Therefore, we isolated a set of mutants resistant to 5-azacytidine and compared the kinetics of metabolic conversion of several nucleosides by mutant and wild-type cells.

MATERIALS AND METHODS

The following non-labeled nucleosides were used: Cytidine, Hoffmann La Roche; adenosine, E. Merck; deoxyadenosine, chemically pure, Fluka; thymidine, A-grade, Calbiochem. 5-Azacytidine was prepared in this institute using the methods described previously[12]. [2-^{14}C]Thymidine (specific activity, 40.5 mCi/mmole), [G-^{14}C]-cytidine (210 mCi/mmole) and [8-^{3}H]adenosine (8 Ci/mmole) were obtained from the Institute for Research and Application of Radioisotopes, Praha. [4-^{14}C]5-Azacytidine (30 mCi/mmole) was prepared in this institute by Ing. J. Kopecký.

Spontaneous mutants resistant to 5-azacytidine were selected as follows: A culture of the parent strain, *E. coli* B, diluted to $5 \cdot 10^5$ cells/ml, was plated on minimal glucose–mineral salts agar[13] containing 10 μg/ml 5-azacytidine. In order to avoid decomposition of 5-azacytidine by heat, this substance was added to the agar medium cooled down to about 50 °C just before pouring the plates. Wild-type bacteria did not form visible colonies on these plates even after 3 days incubation at 37 °C, while strains resistant to 5-azacytidine grew with some delay, forming colonies after about 36 h. The mutants retained their resistant character during many passages on tryptone agar without 5-azacytidine.

The bacteria were grown in shaken flasks at 37 °C on glucose–mineral salts medium[13] supplemented with 0.25 % casamino acids. For measurements of velocities of metabolic conversion of nucleosides the logarithmically growing bacteria at a density of about $3 \cdot 10^8$ cells/ml were filtered on a nitrocellulose membrane filter (Sartorius, diameter 50 mm) and resuspended in fresh medium with glucose but without casamino acids. For most kinetic measurements the density of $5 \cdot 10^8$ cells/ml was suitable; if data at very low substrate concentrations were required, the bacteria were diluted to $5 \cdot 10^7$ cells/ml. All data concerning the maximum reaction velocities (Table I) have been normalized to $1 \cdot 10^8$ cells/ml, assuming a linear relationship between the reaction rate and the number of bacteria per unit volume.

The preparation of T4-phage stocks and the inactivation of phage by osmotic shock has been described elsewhere[14]. For infection 10 plaque-forming units of phage, or 20 disrupted phage particles per bacterial cell were used. 3 min after the addition of phage, samples were taken for determining the number of surviving cells, and the reaction was started by adding the labeled nucleosides.

The rate of incorporation of nucleosides was measured by taking samples (1–2 ml) into an equal volume of 10 % trichloroacetic acid, filtrating the mixture on nitrocellulose membrane filters, and counting on a gas-flow counter. Other types of metabolic conversion of nucleosides were investigated in the following manner: At zero time aliquots of suitably diluted bacterial culture were pipetted into prewarmed shaken small erlenmeyer flasks containing the labeled substrates and inhibitors dissolved in a minimum volume of water. Samples (0.25 ml) were usually taken at 4-min intervals into chilled tubes containing excess of non-labeled substrate and products of the reaction under study. The samples were analyzed by paper chromatography as described previously[11]. In the simplest cases, such as the incorporation of cytidine or 5-azacytidine, the reaction velocity was constant for at least 20 min. The phosphorolysis of thymidine was usually more rapid during the first 3–4 min than afterwards; in such cases the rate between 4 and 20 min after the addition of the substrate was taken as the reaction velocity. The deamination of adenosine[15] in

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the mutant strain took a very complex course due to the rapid exhaustion of the substrate by incorporation into RNA, as well as by further conversion of primarily formed inosine; therefore, meaningful quantitative data about the kinetics of this reaction could not be obtained.

The rate of deamination of 5-azacytidine could not be determined by chromatography since the 5-azauridine formed is very unstable in neutral aqueous solution[16]. 5-Azacytidine and 5-azauridine in acidic media have absorption maxima at 255 and 240 nm, respectively; therefore, qualitative evidence of deamination could be obtained in glucose–mineral salts medium (without amino acids), filtered on nitrocellulose membrane filters to remove bacteria and acidified to pH 1.0 with 2M HCl, measuring the shift of the absorption maxima from 255 nm to shorter wavelengths.

RESULTS

Spontaneous mutants resistant to 5-azacytidine were found to occur in populations of *E. coli* at a frequency of about 10^{-4}. Each of the eighteen resistant strains isolated had approximately the same degree of tolerance of the inhibitor, their growth being partially retarded by 10 μg/ml of the inhibitor in the agar medium. All mutants have been shown to incorporate 5-azacytidine at a reduced rate in comparison with the wild-type strain. One of these mutants was investigated more closely, determining the kinetics of metabolic conversion of various nucleosides by whole cells.

Lineweaver–Burk plots of the concentration dependence of the rates of incorporation of both cytidine and 5-azacytidine showed that the Michaelis constant for the incorporation of both substrates is markedly increased in the mutant when compared with wild-type strain. In addition, the maximum velocity (V) of 5-azacytidine incorporation is about 6 times lower in the mutant than in the wild-type strain (Fig. 1B) while there is no significant difference in the V value of cytidine incorporation (Fig. 1A). Consequently, the resistant mutant incorporates much less 5-aza-

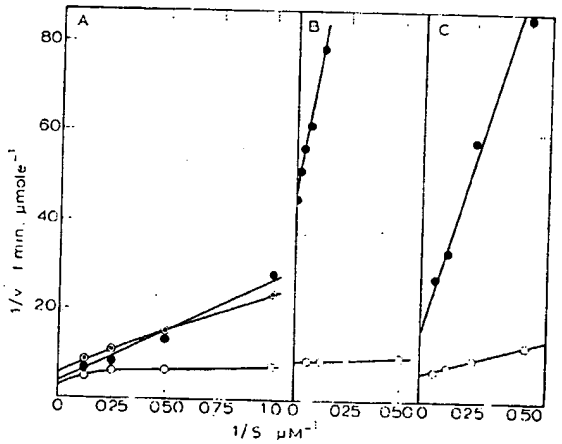


Fig. 1. Kinetics of metabolic conversion of cytidine and 5-azacytidine by strains of *E. coli*, sensitive and resistant to 5-azacytidine. A, incorporation of cytidine; B, incorporation of 5-azacytidine; C, deamination of cytidine. ○—○, *E. coli* B (wild-type strain); ⊙—⊙, *E. coli* B, infected with T_4-phage; ●—●, 5-azacytidine-resistant strain. The density of the bacterial suspension was 5·10^{7} cells/ml.

cytidine than the wild-type strain, irrespective of substrate concentration in the medium; with cytidine a marked difference in the incorporation rates is detectable at concentrations of about 1 μM; when the substrate concentration is increased up to 8 μM the difference becomes less apparent.

The deamination of cytidine is affected in the mutant strain in a similar manner; Fig. 1C shows a considerable increase of the Michaelis constant and some reduction of maximum velocity in the mutant. The kinetics of deamination of 5-azacytidine could not be measured exactly, since the 5-azauridine formed is rapidly decomposed. Spectrophotometric data show, however, that 5-azacytidine is stable in contact with mutant cells, while a gradual shift of absorption maxima at pH 1.0 is observed in the wild-type culture, indicating deamination of 5-azacytidine.

The pleiotropic character of the mutation leading to resistance to 5-azacytidine is illustrated by the fact that phosphorolysis of thymidine, a reaction apparently unrelated to the metabolism of cytidine, was affected in a similar manner; we observed a marked increase of Michaelis constant with little change of maximum velocity (Fig. 2).

Many attempts were undertaken to measure the rates of deamination of adenosine by mutant and wild-type cells; no exactly comparable data could, however, be obtained for reasons explained above; nevertheless the experiments (not shown) indicated that the difference between both strains is much smaller than in the case of deamination of cytidine or phosphorolysis of thymidine.

A peculiar property of mutant cells, *i.e.* the strong impairment of the ability to incorporate 5-azacytidine concurrent with a moderate reduction of cytidine uptake, strikingly resembles the effect of T4-phage infection on wild-type cells[14]. In order to verify the significance of this similarity we repeated some of the foregoing experiments now comparing the intact wild-type bacteria with those infected with T4-phage or treated with disrupted phage. We observed that the kinetics of these reactions was altered by phage; the Lineweaver–Burk plots of infected wild-type cells were nearly superimposable with those of the mutant, but divergent from those of the uninfected culture (Figs 1 and 2). It seems that the mutation had affected the same catalytic

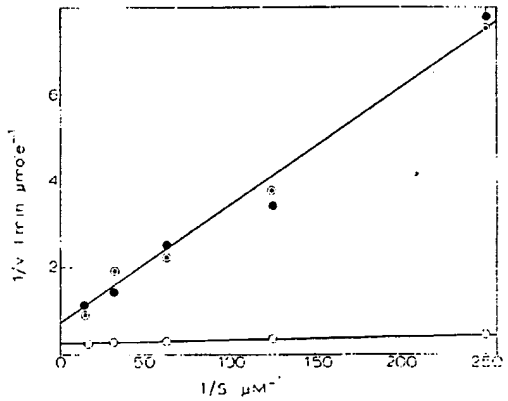


Fig. 2. Kinetics of phosphorolysis of thymidine by strains of *E. coli*, sensitive and resistant to 5-azacytidine. ○—○, *E. coli* B (wild-type strain); ◉ ◉, *E. coli* B, treated with osmotically disrupted T4-phage; ●—●, 5-azacytidine-resistant strain. The density of the bacterial culture was $5 \cdot 10^8$ cells/ml.

component of the cell as does the phage; if this is correct, we would expect that the kinetic characteristics of the mutant would not change any further upon phage treatment. The data of Table I show that the K_m of phosphorolysis of thymidine increases 8.5 times upon treatment of wild-type cells with disrupted phage, while a similar treatment of mutant strain causes a relatively small increase by a factor of 1.5. The observed decrease of V may perhaps be due to blocking of the catabolism of the deoxyribose moiety of thymidine as observed by Munch-Petersen and Vilstrup[17]. It should be noted that the mutant is equally sensitive to T4-phage as the wild-type strain, propagating this phage with equal efficiency.

TABLE I

APPARENT MICHAELIS CONSTANTS AND MAXIMUM REACTION VELOCITIES OF METABOLIC CONVERSION OF CYTIDINE AND 5-AZACYTIDINE BY 5-AZACYTIDINE-SENSITIVE (WILD-TYPE) AND RESISTANT STRAINS OF *E. coli*

The values of maximum reaction velocities have been normalized to a density of bacterial culture equal to $1 \cdot 10^8$ cells/ml.

Substrate	*Reaction*	*Bacteria*	V (*μmoles·min*$^{-1}$·*l*$^{-1}$)	K_m (*μM*)
Cytidine	Incorporation	Wild-type	0.36*	0.25
	Incorporation	Resistant	0.56	7.1
	Incorporation	Wild-type, T4-infected	0.42	4.2
5-Azacytidine	Incorporation	Wild-type	0.28	2.1
	Incorporation	Resistant	0.05	20
Cytidine	Deamination	Wild-type	0.34	2.3
	Deamination	Resistant	0.14	8.7
Thymidine	Phosphorolysis	Wild-type	0.83	4.0
	Phosphorolysis	Resistant	0.27	34
	Phosphorolysis	Wild-type, T4-infected	0.27	34
	Phosphorolysis	Resistant, T4-infected	0.15	50

* Value obtained by extrapolating the less steep straight line (Fig. 1A) to zero reciprocal substrate concentration.

The foregoing experiments indicate that the mutation and the treatment with T4-phage affect a high-affinity component common to incorporation or deamination of cytidine and 5-azacytidine as well as to the phosphorolysis of thymidine.

In wild-type cells many types of metabolic conversion of nucleosides are competitively inhibited by structurally and metabolically unrelated nucleosides[5,6,11] and it was of interest to investigate whether the low-affinity conversions in mutant bacteria are still susceptible to this kind of inhibition. Therefore, the competition experiments were repeated with the mutant strain, and the results were expressed in terms of the K_m/K_I ratios (Table II), calculated from the Lineweaver–Burk plots as earlier described[11]. Fig. 3 shows that 5-azacytidine as well as cytidine are capable of competitively inhibiting the phosphorolysis of thymidine in the mutant; the K_m/K_I ratios are close to unity; in fact, 5-azacytidine is slightly more active than cytidine, in spite of the very limited rate of uptake of the former. This finding again confirms the parallelism between the mutated and T4-phage-infected cells, where 5-azacytidine

TABLE II

THE VALUES OF K_m/K_i OF NUCLEOSIDE CONVERSION INHIBITED BY HETEROLOGOUS NUCLEOSIDES

Substrate	*Reaction*	*Inhibitor*	K_m/K_i	
			Wild-type bacteria*	*Mutant bacteria*
Thymidine	Phosphorolysis	Cytidine	n.d.**	1.03
	Phosphorolysis	5-Azacytidine	3.5	1.23
5-Azacytidine	Incorporation	Adenosine	1.32	1.34
	Incorporation	Deoxyadenosine	n.d.**	0.45
	Incorporation	Thymidine	0.25	0.24

* The values for wild-type bacteria are taken from ref. 11.
** n.d., not determined.

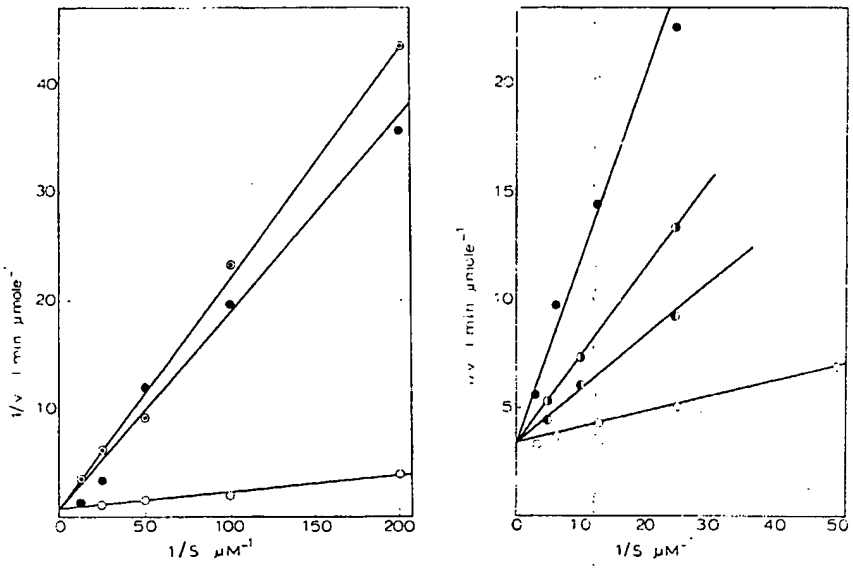


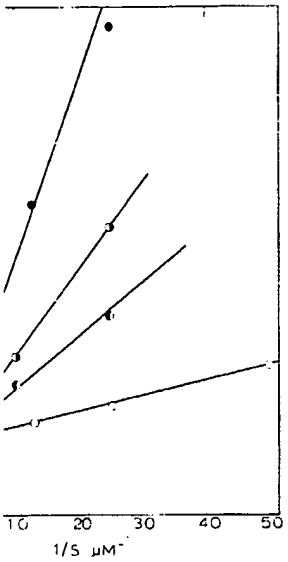
Fig. 3. Inhibition of phosphorolysis of thymidine in 5-azacytidine-resistant mutant by cytidine and 5-azacytidine. ○—○, thymidine alone; ●—●, cytidine; ◉--◉, 5-azacytidine. The concentration of competing nucleosides was 200 μM. The density of the bacterial cultures was $5 \cdot 10^8$ cells/ml.

Fig. 4. Inhibition of incorporation of 5-azacytidine in 5-azacytidine-resistant mutant by heterologous nucleosides. ○···○, 5-azacytidine alone; ●—●, adenosine; ◑—◑, deoxyadenosine; ◐—◐, thymidine. The concentration of the competing nucleosides was 200 μM. The density of bacterial culture was $7.5 \cdot 10^8$ cells/ml.

retains its ability to inhibit the phosphorolysis of thymidine in spite of selective impairment of its uptake[18,19]. The residual incorporation of 5-azacytidine by the mutant strain is still competitively inhibited by adenosine and less efficiently by deoxyadenosine and thymidine (Fig. 4).

) BY HETEROLOGOUS NUCLEOSIDES

K_m/K_i	
Wild-type bacteria*	*Mutant bacteria*
n.d.**	1.03
3.5	1.23
1.32	1.34
n.d.**	0.45
0.25	0.24



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DISCUSSION

Selecting for resistance to 5-azacytidine, a strain of *E. coli* was obtained with altered kinetics of metabolic conversion of nucleosides, involving at least three distinct enzymes, namely cytidine kinase (EC 2.7.1.48), cytidine deaminase (EC 3.5.4.5) and thymidine phosphorylase (EC 2.4.2.4). Except for a common nucleoside or deoxynucleoside structure of substrates, these reactions are apparently unrelated; the impairment of the rate of incorporation of 5-azacytidine cannot be due to a lack of deamination, since we know that strains deficient in cytidine deaminase incorporate 5-azacytidine with nearly the same efficiency as wild-type strains[20]. All enzymes are still functional in the mutant and probably also in T4-treated cells, since the reactions catalyzed by them proceed at sufficiently high concentrations of the substrates. Munch-Petersen and Vilstrup[17] measured the activities of enzymes of thymidine catabolism in cell-free extracts of bacteria before and after infection with T4-phage or treatment with phage ghosts; no decrease of the activity of thymidine phosphorylase was found. It seems that the mutation as well as the phage treatment affect a catalytic component common to all these enzymic conversions, which is most likely to be a permease mediating the transport of several nucleosides and deoxynucleosides. The existence of such a permease has been anticipated on the basis of mutual competition of heterologous nucleosides[5,6]. The present experiments show, however, that even if the high-affinity component is missing, the residual low-affinity reactions are still susceptible to competitive inhibition by heterologous nucleosides. Therefore, wild-type bacteria seem to contain two different nucleoside permeases with different affinity for the substrates. The high-affinity component is inactivated by the mutation or T4-phage treatment, while the low-affinity component remains operative. Both these permeases are common for a number of different nucleosides and deoxynucleosides; no permease specific for a single nucleoside has been found so far.

It may be objected that neither the competition of heterologous nucleosides nor the pleiotropic character of a mutant necessarily indicate the involvement of permeases. Hammer-Jespersen *et al.*[21] found that adenosine or cytidine induce the synthesis of thymidine-catabolizing enzymes; no explanation has been proposed. This remarkable finding shows that apparently independent pathways of nucleoside metabolism may actually be linked by unknown catalytic or regulatory components. In case of our mutant, however, in which the kinetics of different types of metabolic conversion is affected in an analogous manner, the permease hypothesis seems to be the most acceptable.

The existence of two distinct permeases enables us to explain the differential effect of the mutation or phage treatment on the incorporation of cytidine and 5-azacytidine. We have seen that the high-affinity component of cytidine incorporation is very limited, being detectable at very low substrate concentrations only; at higher concentrations most of the cytidine incorporated is transported by the low-affinity component. The opposite is true with 5-azacytidine; its transport by the low-affinity system is very inefficient and practically all uptake in the wild-type strain is due to the high-affinity permease. Therefore, the inactivation of the latter strongly affects the incorporation of 5-azacytidine but has little effect on the incorporation of cytidine, unless measured at very low substrate concentration. The heterogeneity of cytidine transport in *E. coli* has been postulated earlier by Peterson *et al.*[5]. It is remarkable

that 5-azacytidine very efficiently inhibits the transport mediated by the low-affinity permease but that its own transport by this permease is severely limited. No definite explanation for this behaviour can be proposed at present. Many examples of this type of interaction are known from studies of the permeases of amino acids[22].

We may ask whether the two permease components are acting independently in parallel or whether they operate in a consecutive manner, forming an integrated system as proposed by Koch[3].

According to Peterson and Koch[6] the nucleoside-transporting system consists of a binding site, a permease and possibly a component mediating the transfer of energy. The histidine-transporting system in *Salmonella typhimurium* has been shown[23] to consist of two distinct independent binding proteins of high affinity, and a permease; an additional, less specific transport system may participate in histidine uptake, working in parallel with the main histidine-specific system. The relationship between the two nucleoside-transporting components cannot be determined with certainty until mutants deficient in the low-affinity component become available; obviously such mutants cannot be expected to occur among 5-azacytidine-resistant strains; slowly growing cytidine auxotrophs should be promising for the isolation of this kind of mutants.

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